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**PATENT**

Attorney Docket No. INL-036DV

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

| | | | |
|---|---|---|---|
| APPELLANT(S): | Dahlbäck | | |
| SERIAL NO.: | 09/912,947 | GROUP NO.: | 1634 |
| FILING DATE: | July 25, 2001 | EXAMINER: | Bausch, Sarae L. |
| TITLE: | *Assays for Determining Anticoagulant Cofactor Activity* | | |

Mail Stop Appeal Brief-Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

## REPLY BRIEF

Sir:

This Reply Brief is submitted in response to the Examiner's Answer mailed from the U.S. Patent and Trademark Office on September 1, 2006. Appellant believes no fee is necessary for consideration of this paper. However, if a fee is required, please consider this a conditional petition therefor and authorization to debit Deposit Account No. 50-1721.

## I. REAL PARTY IN INTEREST

The Real Party in Interest is provided in the Brief in Support of An Appeal ("Appeal Brief") filed on June 14, 2006.

## II. RELATED APPEALS AND INTERFERENCES

Appellant wishes to make the Board aware that the parent of the present application, U.S. Serial No. 08/500,917, has undergone the following interference proceedings.

| | |
|---|---|
| Patent Interference No. 105,235 | Griffin *et al.* (Patent 5,705,395)<br>v.<br>Dahlbäck (USSN 08/500,917) |
| Patent Interference No. 105,268 | Griffin *et al.* (Patent 5,834,223)<br>v.<br>Dahlbäck (USSN 08/500,917) |
| Patent Interference No. 105,269 | Griffin *et al.* (Patent 6,083,757)<br>v.<br>Dahlbäck (USSN 08/500,917) |

The decision of no-interference-in-fact was rendered in each of the three interference proceedings identified above on June 30, 2006. Copies of the decisions are provided in the attached Related Proceedings Appendix.

## III. STATUS OF THE CLAIMS

The status of the claims is provided in the Appeal Brief filed on June 14, 2006. The Examiner's Answer has acknowledged that the status of the claims contained in the brief is correct.

## IV. STATUS OF AMENDMENTS

The status of amendments after final is provided in the Appeal Brief filed on June 14, 2006. The Examiner's Answer has acknowledged that the statement of the status of amendments after final contained in the brief is correct.

## V. SUMMARY OF THE CLAIMED SUBJECT MATTER

The summary of the claimed subject matter is provided in the Appeal Brief filed on June 14, 2006. The Examiner's Answer has acknowledged that the summary of the claimed subject matter contained in the brief is correct.

## VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

One ground of rejection raised by the Examiner in the Final Office Action and the Examiner's Answer is to be reviewed on appeal. That is the rejection of claims 46, 53-55, 64 and 65 under 35 U.S.C. § 112, first paragraph, as failing to comply with the enablement requirement.

## VII. APPELLANT'S ARGUMENT

In the Examiner's Answer, the Examiner maintained the rejection of claims 46, 53-55, 64 and 65 under 35 U.S.C. § 112, first paragraph, for allegedly failing to enable one skilled in the art to make and/or use the invention without undue experimentation. Appellant has responded to the rejection originally raised in the Final Office Action in the Appeal Brief filed on June 14, 2006. Appellant hereby incorporates by references section VII, Appellant's Argument, presented in the Appeal Brief filed on June 14, 2006, in its entirety.

The Examiner noted, on page 20 of the Examiner's Answer, that Shen *et al.* (Evidence Appendix G submitted together with the Appeal Brief on June 14, 2006) is a post-filing art since Shen *et al.* was published in April 1993 while the priority documents for the present application were filed in January 1993. Appellant submits that two priority documents were filed in this application, *i.e.*, Swedish Application No. 9300300-2, filed January 29, 1993, and Swedish Application No. 9302457-8, filed July 20, 1993. Appellant submits that the invention claimed in claims 46, 53-55, 64 and 65 is entitled to the priority date of July 20, 1993.

In this Reply Brief, Appellant addresses new ground of rejection raised in the Examiner's Answer.

In response to Appellant's argument presented in the Appeal Brief, the Examiner relied on three post-filing date references, namely, Price *et al.* (1997) Ann. Intern. Med., 127:895-903; de Visser *et al.*, (2000) Thromb. Haemost., 83:577-82; and Bertina R. (2001) Thromb. Haemost.,

86:92-103, to allege that the specification does not give any guidance on how to predictably correlate any abnormal presence or absence of a nucleic acid fragment or sequence of the Factor V gene with any particular types or all types of thrombosis in an individual. In particular, the Examiner asserts that Price *et al.* (1997) demonstrates that even if a mutation in the Factor V gene is found to be associated with one type of thrombosis, it does not predictably correlate that mutation with all types of thrombosis in all individuals. See, *e.g.*, the Examiner's Answer, pages 9 and 15. The Examiner further asserts that de Visser *et al.* (2000) teaches that the HR2 haplotype of the Factor V gene was not found to be associated with an increased risk in a population-based case study for venous thrombosis. See, *e.g.*, the Examiner's Answer, page 8. The Examiner also asserts that Bertina (2001) teaches that the Factor V Leiden mutation is a risk factor for deep-vein thrombosis, cerebral vein thrombosis, superficial vein thrombosis, and portal vein thrombosis but not for primary pulmonary embolism and retinal vein thrombosis. See, *e.g.*, the Examiner's Answer, page 15. The Examiner then pointed to Table 2 in Bertina alleging that Bertina teaches that two mutations present in the Factor V gene are not associated with either APC-resistance or increase in thrombosis risk. See, *e.g.*, the Examiner's Answer, page 15. Therefore, the Examiner concluded that it would require undue experimentation to practice the claimed invention because the post-filing art teaches that mutations found in the Factor V gene are not correlative to all types of thrombosis as the claimed invention broadly encompasses. See, *e.g.*, the Examiner's Answer, pages 11, 12 and 22. A copy of Price *et al.* is enclosed as Evidence Appendix A. A copy of de Visser *et al.* is enclosed as Evidence Appendix B. A copy of Bertina is enclosed as Evidence Appendix C.

The test for enablement is whether one reasonably skilled in the art could make or use the invention as broadly as it is claimed based on the disclosures in the specification coupled with information known in the art without undue experimentation. See *In re* Wands, 858 F.2d 731 (Fed. Cir. 1988). The state of art existing at the filing date must be used to determine the enablement. See, *e.g.*, MPEP § 2164.05(a). The United States Court of Appeals for the Federal Circuit has held that this principle applies to applicant as well as to the PTO. See, *In re* Glass, 492 F.2d 1228, 1232 (CCPA 1974); and *In re* Hogan, 559 F.2d 595 (Fed. Cir. 1977). Under this principle, the Examiner may not use the post-filing date references to demonstrate that the invention is not enabled unless a later-dated reference provides evidence of what one skilled in

the art would have known on or before the effective filing date or if a later-dated reference provides evidence that the disclosed invention was not possible at the time of filing. MPEP § 2164.05(a) citing *In re* Hogan, 559 F.2d 595 (Fed. Cir. 1977) and *In re* Wright, 999 F.2d 1557 (Fed. Cir. 1993).

Furthermore, the Federal Circuit stated in Hogan that: "Rejections under s 112, first paragraph, on the ground that the scope of enablement is not commensurate with the scope of the claims, orbit about the more fundamental questions: To what scope of protection is this applicant's particular contribution to the art entitled?" *In re* Hogan, 559 F.2d 595 (Fed. Cir. 1977). In Hogan, the Federal Circuit faced the question whether a generic claim for solid polymers of olefins may issue of breath sufficient to encompass the later existing "non-enabled" amorphous polymers. *Id.* The Hogan court recognized the "pioneer" status of the appellants' invention because "[t]he record reflects no citation of prior art disclosing a solid polymer of 4-methyl-1-pentene, which may suggest that appellants at least broke new ground in a broad sense." The court held that: "As pioneers, if such they be, they would deserve broad claims to the broad concept. What were once referred to as 'basic inventions' have led to 'basic patents,' which amounted to real incentives, not only to invention and its disclosure, but to its prompt, early disclosure. If later states of the art could be employed as a basis for rejection under 35 U.S.C. s 112, the opportunity for obtaining a basic patent upon early disclosure of pioneer inventions would be abolished." *Id.*

At the outset, Appellant wishes to emphasize that the present application describes a truly pioneer invention, which is the discovery for the first time ever that Factor V has a novel anticoagulant activity and the deficiency of such activity causes Activated Protein C (APC) resistance and associated thrombosis. This discovery directly linked the Factor V gene to APC-resistance and risk for thrombosis for the first time. The file history reflects no citation of prior art disclosing a linkage between the Factor V gene and APC-resistance or risk for thrombosis. In fact, the Examiner repeatedly stated in the Final Office Action and the Examiner's Answer that "[t]he prior art does not provide any genetic variations within the Factor V gene that are associated with thrombosis or APC resistance." See, *e.g.*, the Examiner's Answer, page 6.

The significance of this discovery was apparent from a statement by Voorberg *et al.* in The Lancet, 343:1535-1536, published in June 18, 1994, reporting determination of a particular mutation in the Factor V gene responsible for thromboembolism associated with APC-resistance based on this discovery. At the beginning of the Lancet paper, Voorberg *et al.* stated:

> Venous thromboembolism has been associated with molecular defects in several haemostatic components: antithrombin II, protein C, protein S, plasminogen, and fribrinogen.[1] However, in over 90% of patients the cause remains obscure.[2] A poor anticoagulant response to activated protein C (APC) has been observed in about 20-30% of patients with an idiopathic predisposition to thromboembolic disease.[3,6] This abnormal response has been linked to a plasma factor which appeared to be identical to coagulation factor V.[7] These observations suggest that a molecular abnormality in factor V underlies the thrombotic events that are associated with a defective anticoagulant response to APC in vitro." [Emphasis added.]

Voorberg *et al.* cited as reference 7, Dahlbäck B. *et al.*, February, 1994, "Inherited resistance to activated protein C is corrected by anticoagulant cofactor activity found to be a property of factor V," PNAS USA, 91:1396-1400, which summarizes the core discovery of the present application and was published in February, 1994, soon after the present application was filed. A copy of Voorberg *et al.* is enclosed as Evidence Appendix D. Evidence Appendix D was initially submitted together with a response to an Office Action on September 28, 2005, and was entered in the record by the Examiner. Evidence Appendix D was also submitted as Evidence Appendix H together with the Appeal Brief filed on June 14, 2006.

Therefore, contrary to the Examiner's statement in the Examiner's Answer that "[t]he instant application fails to add to the state of the art because it does not teach any mutations or polymorphisms within the human Factor V gene," Appellant submits that the invention described in the present application broke a whole new ground in the field of thrombosis diagnosis and treatment as acknowledged by skilled artisans like Voorberg *et al.* The prompt, early disclosure of this discovery by filing the present application allowed skilled artisans like Voorberg *et al.* to immediately determine a particular mutation at position Arg506 in the Factor V protein responsible for thromboembolism associated with APC-resistance without undue experimentation. See, the Appeal Brief filed on June 14, 2006, pages 14-15. The prompt, early disclosure of this discovery also permitted later inventors and scientists such as Price *et al.*, de Visser *et al.*, and Bertina, to further improve the invention by identifying and characterizing

specific natures of different types of mutations in the Factor V gene and their associations with particular types of thrombosis.

As discussed above, the Federal Circuit stated, in Hogan, that pioneer inventions deserve broad claims to the broad concept. Hogan, 559 F.2d 595 (Fed. Cir. 1977). The Hogan court further emphasized that conferring broad protections to pioneer inventions amounted to real incentives, not only to invention and its disclosure, but to its prompt, early disclosure. *Id.* If later states of the art could be employed as a basis for the enablement rejection, the opportunity for obtaining a basic patent upon early disclosure of pioneer inventions would be abolished. *Id.* Appellant submits that the invention described in the present application is precisely the type of pioneer invention that the Hogan court recognized and deemed to deserve broad protection. Appellant respectfully urges the Board to follow the Hogan court to recognize the pioneer status of the present invention that deserves broad claims to the broad concept.

Thus, as a threshold matter, Appellant submits that the post-dated references, Price *et al.*, de Visser *et al.*, and Bertina, can not be used for the enablement rejection because, as clear from the Examiner's Answer, these references were not used to provide evidence of what one skilled in the art would have known on or before the effective filing date of the present application and, as argued below, none of the cited references provide any evidence that the invention as claimed in claims 46, 53-55, 64 and 65 was not possible at the time of filing.

**Claims 46, 53, 64 and 65**

As argued in the Appeal Brief filed on June 14, 2006, Appellant respectfully submits that the present application fully enables one reasonably skilled in the art to make or use the invention as claimed in claims 46, 53, 64 and 65 based on the disclosures in the specification coupled with knowledge known in the art at the time of filing without undue experimentation. For the reasons enumerated below, Appellant further submits that none of the cited post-filing references, Price *et al.*, de Visser *et al.*, and Bertina, provide any evidence that the invention as claimed in claims 46, 53, 64 and 65 was not possible at the time of the filing.

Independent claim 46 recites:

> 46. A method for detecting an individual at risk of developing thrombosis, said method comprising:

(a) obtaining a sample from the individual;

(b) conducting a nucleic acid assay on the sample, wherein the nucleic acid assay is a hybridization assay or a sequencing assay;

(c) determining abnormal presence or absence of at least one nucleic acid fragment or sequence in the individual's Factor V gene compared to a normal control; and

(d) detecting the individual at risk of developing thrombosis based on the determination of step (c).

[Emphasis added.]

Thus, claim 46 only requires detecting an individual who is more likely to develop thrombosis, any type of thrombosis, than a normal individual during life by detecting abnormal presence or absence of at least one nucleic acid fragment or sequence in the individual's Factor V gene compared to a normal control. Claim 46 does not require detecting an individual who will develop thrombosis with 100% certainty. Claim 46 also does not require detecting an individual at risk of developing a particular type of thrombosis. Nor does claim 46 require detecting an individual at risk of developing all types of thrombosis. In another word, claim 46 does not require association of one particular abnormal sequence in the Factor V gene with one particular type of thrombosis, such as, deep-vein thrombosis, cerebral vein thrombosis, superficial vein thrombosis, portal vein thrombosis, primary pulmonary embolism, or retinal vein thrombosis. Claim 46 also does not require association of one particular abnormal sequence in the Factor V gene with all types of thrombosis as described above. Such associations come into existence later in the art and are improvement inventions based on the disclosure of the present invention. Claim 46, on the other hand, is directed to the seminal discovery of the direct link between the Factor V gene and APC-resistance and increased risk for thrombosis.

As the Examiner pointed out, Price *et al.*, de Visser *et al.*, and Bertina describe efforts to associate specific mutations or polymorphisms in the Factor V gene identified later in the art with particular types of thrombosis, a state of art coming into existence after the filing date of the present application. None of Price *et al.*, de Visser *et al.*, and Bertina provide any evidence that it would be impossible to detect an individual who is more likely to develop thrombosis, any type of thrombosis, than a normal individual by detecting abnormal presence or absence of at least one nucleic acid fragment or sequence in the individual's Factor V gene compared to a

normal control, as claimed in claim 46. In fact, Price *et al.*, de Visser *et al.*, and Bertina, each provides evidence that the invention claimed in claim 46 is fully enabled because each of the cited references demonstrates that an abnormal sequence (*i.e.*, a mutation or a polymorphism) in the Factor V gene was found to be associated with at least one type of thrombosis in at least some populations. For example, Bertina teaches, as the examiner acknowledged, that the Factor V Leiden mutation (Arg506Gln) is a risk factor for deep-vein thrombosis, cerebral vein thrombosis, superficial vein thrombosis, and portal vein thrombosis. Contrary to the Examiner's assertion that Bertina teaches two mutations in the Factor V gene shown in Table 2, Arg485Lys and His1299Arg, not associated with either APC-resistance or increase in thrombosis risk, Bertina clearly indicates that mutation Arg485Lys was found to be likely associated with APC-resistance by at least one research group and mutation His1299Arg was found to be associated with increased risk of thrombosis by at least two research groups. *See*, Bertina, Table 2. Similarly, Price *et al.* teaches that the Factor V Leiden mutation is responsible for most cases of resistance to activated protein C. See, Price *et al.*, page 895, right column. In addition, de Visser *et al.* acknowledged that the HR2 haplotype of the Factor V gene was found to be associated with increased risk for venous thrombosis by a French study group even though de Visser *et al.* did not find such association in its own population-based study. See, *e.g.*, de Visser *et al.*, page 577, left column, and in the Discussion section.

Therefore, Appellant submits that the evidence provided in Price *et al.*, de Visser *et al.*, and Bertina further support that detection of a mutation manifested by at least one abnormal nucleic acid fragment or sequence in an individual's Factor V gene is sufficient to indicate that the individual is more likely to develop thrombosis, *e.g.*, venous thrombosis or any other types of thrombosis, than a normal individual during life time. As Bertina stated, thrombosis is a complex disease, in which multiple biological pathways contribute to the risk of developing the disease. For example, "[p]resent models of venous thrombotic risk hypothesize that a clinical event will occur only when the 'thrombosis potential'-which is a function of age, genetic and environmental factors and their interactions (additive or synergistic)-has passed a certain threshold." See, Bertina, page 92, left column. Applying the Bertina theory, Appellant submits that detection of an abnormal nucleic acid fragment or sequence in an individual's Factor V gene

is a genetic factor that increases the "thrombosis potential," which, in turn, leads to increased risk for the actual occurrence of a thrombotic event.

For at least the above reasons and the arguments presented in the Appeal Brief filed on June 14, 2006, Appellant respectfully submits that the present application fully complies with the enablement requirement with respect to independent claim 46 and its dependent claims 53, 64 and 65 and request reconsideration and withdrawal of the rejection of claims 46, 53, 64 and 65 under 35 U.S.C. § 112, first paragraph.

**Claims 54 and 55**

As argued in the Appeal Brief filed on June 14, 2006, Appellant respectfully submits that the present application fully enables one reasonably skilled in the art to make or use the invention as claimed in claims 54 and 55 based on the disclosures in the specification coupled with knowledge known in the art at the time of filing without undue experimentation. For the reasons enumerated below, Appellant further submits that none of the cited post-filing references, Price *et al.*, de Visser *et al.*, and Bertina, provides any evidence that the invention as claimed in claims 54 and 55 was not possible at the time of the filing date.

Independent claim 54 recites:

> 54. A method for determining a presence of a Factor V gene mutation associated with Activated Protein C (APC)-resistance in an individual at risk for APC-resistance, the method comprising the steps of:
>
> (a) obtaining a sample from the individual;
>
> (b) conducting a nucleic acid sequencing assay on the sample using reagents specific for the Factor V gene to determine the Factor V gene sequence; and
>
> (c) determining the presence of the Factor V gene mutation associated with APC-resistance in the individual by comparing the sequence of the Factor V gene from step (b) to a normal Factor V gene sequence.
> [Emphasis added.]

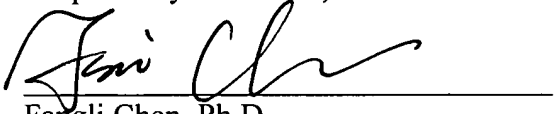
Thus, claim 54 relates to a method for determining a presence of Factor V gene mutation associated with APC-resistance in an individual at risk for APC-resistance. Claim 54 requires obtaining a sample from an individual known to have APC-resistance or be at risk for APC-resistance, conducting a nucleic acid sequencing assay on the sample using reagents specific for

the Factor V gene to determine the Factor V gene sequence and determining the presence of the Factor V gene mutation associated with APC-resistance in the individual by comparing the determined sequence to a normal Factor V gene sequence.

As discussed above, Price *et al.*, de Visser *et al.*, and Bertina discuss association of specific mutations or polymorphisms in the Factor V gene identified later in the art with particular types of thrombosis, a state of art coming into existence after the filing date of the present application. None of Price *et al.*, de Visser *et al.*, and Bertina provide any evidence that it would be impossible to obtain a sample from an individual known to have APC-resistance or be at risk for APC-resistance, to conduct a nucleic acid sequencing assay on the sample using reagents specific for the Factor V gene to determine the Factor V gene sequence and to determine the presence of the Factor V gene mutation associated with APC-resistance in the individual by comparing the determined sequence to a normal Factor V gene sequence, as claimed in claim 54. In fact, as argued in the Appeal Brief filed on June 14, 2006, those skilled in the art (*e.g.*, Voorberg *et al.*) were able to determine a particular mutation in the Factor V gene that caused APC-resistance based upon the disclosure of the present application coupled with the knowledge in the art, *e.g.*, the sequence of Factor V gene and methods of sequencing, without undue experimentation. See, the Appeal Brief, pages 14 and 15, and Voorberg *et al.*

For at least the above reasons and the arguments presented in the Appeal Brief filed on June 14, 2006, Appellant respectfully submit that the present application fully complies with the enablement requirement with respect to independent claim 54 and its dependent claim 55 and request reconsideration and withdrawal of the rejection of claims 54 and 55 under 35 U.S.C. § 112, first paragraph.

Respectfully submitted,



Date: November 1, 2006
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## CLAIMS APPENDIX

1-45. (Canceled)

46. (Previously presented) A method for detecting an individual at risk of developing thrombosis, said method comprising:

(a) obtaining a sample from the individual;

(b) conducting a nucleic acid assay on the sample, wherein the nucleic acid assay is a hybridization assay or a sequencing assay;

(c) determining abnormal presence or absence of at least one nucleic acid fragment or sequence in the individual's Factor V gene compared to a normal control; and

(d) detecting the individual at risk of developing thrombosis based on the determination of step (c).

47-52. (Canceled)

53. (Previously presented) The method of claim 46, wherein the nucleic acid assay is a sequencing assay.

54. (Previously presented) A method for determining a presence of a Factor V gene mutation associated with Activated Protein C (APC)-resistance in an individual at risk for APC-resistance, the method comprising the steps of:

(a) obtaining a sample from the individual;

(b) conducting a nucleic acid sequencing assay on the sample using reagents specific for the Factor V gene to determine the Factor V gene sequence; and

(c) determining the presence of the Factor V gene mutation associated with APC-resistance in the individual by comparing the sequence of the Factor V gene from step (b) to a normal Factor V gene sequence.

55. (Previously presented) The method of claim 54, wherein the mutation is determined as an abnormal absence or presence of at least one nucleotide sequence in the Factor V gene.

56-63. (Canceled)

64. (Previously presented) The method of claim 53, wherein the sequencing assay comprises sequencing the Factor V gene using reagents specific for the Factor V gene.

65. (Previously presented) The method of claim 64, wherein the detecting step detects an abnormal nucleotide sequence in the Factor V gene.

## EVIDENCE APPENDIX

This appendix includes copies of the following evidence:

1. Evidence Appendix A relied upon by the Examiner in the Examiner's Answer as to the new ground of rejection to be reviewed on appeal.

2. Evidence Appendix B relied upon by the Examiner in the Examiner's Answer as to the new ground of rejection to be reviewed on appeal.

3. Evidence Appendix C relied upon by the Examiner in the Examiner's Answer as to the new ground of rejection to be reviewed on appeal.

4. Evidence Appendix D submitted together with a response to an Office Action on September 28, 2005, and entered in the record by the Examiner. Evidence Appendix D also submitted as Evidence Appendix H together with the Appeal Brief filed on Jun 14, 2006.

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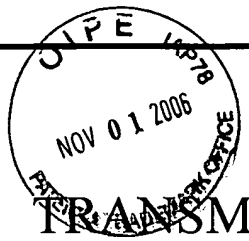


# TRANSMITTAL FORM

| | |
|---|---|
| Application Serial Number | 09/912,947 |
| Filing Date | July 25, 2001 |
| First Named Inventor | Dahlbäck |
| Group Art Unit | 1634 |
| Examiner Name | Bausch, Sarae L. |
| Attorney Docket No. | INL-036DV |
| Patent No. | Not applicable |
| Issue Date | Not applicable |

**ENCLOSURES** *(check all that apply)*

- ☐ Fee Transmittal Form
  - ☐ Check Attached
  - ☐ Copy of Fee Transmittal Form
- ☐ Amendment/Response
  - ☐ Preliminary
  - ☐ After Final
  - ☐ Affidavits/declaration(s)
  - ☐ Letter to Official Draftsperson including Drawings [Total Sheets _____]
- ☐ Petition for Extension of Time
- ☐ Information Disclosure Statement
  - ☐ Form PTO-1449
  - ☐ Copies of IDS Citations
- ☐ Certified Copy of Priority Document(s)
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  - ☐ Statement verifying identity of above
- ☐ Copy of Notice to File Missing Parts of Application
- ☐ Formal Drawing(s)
- ☐ Request For Continued Examination (RCE) Transmittal
- ☐ Power of Attorney (Revocation of Prior Powers)
- ☐ Terminal Disclaimer
- ☐ Executed Declaration and Power of Attorney for Utility or Design Patent Application
- ☐ Small Entity Statement
- ☐ CD(s) for large table or computer program
- ☐ Amendment After Allowance
- ☐ Request for Certificate of Correction
  - ☐ Certificate of Correction (in duplicate)
- ☐ Notice of Appeal to Board of Patent Appeals and Interferences
- ☒ Reply Brief
  - ☒ Claims Appendix
  - ☒ Evidence Appendix A-D
  - ☒ Related Proceedings Appendix
- ☐ Status Inquiry
- ☒ Return Receipt Postcard
- ☐ Certificate of First Class Mailing under 37 C.F.R. 1.8
- ☐ Certificate of Facsimile Transmission under 37 C.F.R. 1.8
- ☐ Additional Enclosure(s) *(please identify below)*

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Evidence Appendix A

REVIEW

# Factor V Leiden Mutation and the Risks for Thromboembolic Disease: A Clinical Perspective

Daniel T. Price, MD, and Paul M. Ridker, MD

**Background:** A single point mutation in the gene coding for coagulation factor V results in a form of factor Va that is resistant to degradation by activated protein C and leads to a relative hypercoagulable state. This mutation, factor V Leiden, is found in 4% to 6% of the U.S. population.

**Purpose:** To review clinical data on factor V Leiden mutation, with emphasis on prevalence of and risks for thromboembolism and implications for screening and management.

**Data Sources:** A MEDLINE search of the English-language literature published between 1993 and April 1997 and an extensive bibliography review.

**Study Selection:** Case–control and prospective cohort studies were reviewed if clinical features of thromboembolic disease associated with factor V Leiden mutation or resistance to activated protein C were presented. Original research articles were reviewed if they addressed the identification of the laboratory abnormality of activated protein C or factor V Leiden mutation. Case reports and case series were reviewed when no analytic data were available.

**Data Extraction:** Review of the identified articles.

**Data Synthesis:** Factor V Leiden mutation is associated with three- to sixfold increases in risks for primary and recurrent venous thromboembolism, especially in patients without transient risk factors, such as surgery or trauma. Risks for venous thromboembolism in genetically affected persons are substantially higher among patients with coexistent predispositions for thrombosis, such as advanced age, use of oral contraceptives, hyperhomocystinemia, and deficiencies of protein C and protein S. Factor V Leiden mutation does not seem to increase risks for arterial thrombosis. Whether patients with the mutation would benefit from more intense or prolonged anticoagulation is unknown.

**Conclusions:** The presence of factor V Leiden mutation predisposes patients to venous thromboembolism, but screening for this disorder is of uncertain utility. Decisions about whether to screen for the mutation will depend on the results of clinical trials designed to evaluate the benefit-to-risk ratio of long-term anticoagulation in the secondary prevention of venous thromboembolism in patients with resistance to activated protein C.

This paper is also available at http://www.acponline.org.

*Ann Intern Med.* 1997;127:895-903.

From Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts. For current author addresses, see end of text.

Thrombophilia is an increased propensity to form thrombosis in the arterial or venous circulation (1, 2). When patients have thromboembolic disease at a young age, have recurrent thromboembolism, or have a strong family history of thromboembolism, physicians often begin a laboratory workup for thrombophilia. The evaluation usually includes a search for inherited disorders of hemostasis by measurement of antithrombin III, protein C, and protein S levels, as well as qualitative and quantitative assays for fibrinogen (1–4). Acquired abnormalities, such as lupus anticoagulant and antiphospholipid antibody, are also often included in the assessment (1, 2). In most cases, however, no defect is identified.

In 1993, investigators in Sweden and the Netherlands described a novel defect in the hemostatic pathway, referred to as resistance to activated protein C (5–7). A single adenine-for-guanine point mutation in the gene coding for coagulation factor V leads to the replacement of arginine by glutamine at position 506. This site is one of the three cleavage sites on factor V for the natural anticoagulant activated protein C. This mutation, which is known as factor V Leiden, makes the activated form of factor V (Va) relatively resistant to degradation by activated protein C. This, in turn, leads to the laboratory abnormality of resistance to activated protein C (**Figure 1**). Factor V Leiden mutation is responsible for most cases of resistance to activated protein C (8–13).

Clinical studies indicate that factor V Leiden mutation is associated with increased risks for primary (7, 9, 14, 15) and recurrent (16, 17) venous thromboembolism, for venous thrombosis during use of oral contraceptives (18–21) and pregnancy (21–23), and for thrombosis in the presence of other genetic and acquired abnormalities of anticoagulation (24–29). On the basis of these observations, screening programs for factor V Leiden mutation have been proposed (21, 30, 31). However, when deciding whether to evaluate a patient for factor V Leiden mutation, one must consider the prevalence of the mutation, the absolute and relative risks imparted by the mutation, the characteristics of available tests, and the benefit-to-risk ratios of any therapeutic intervention based on a positive test finding.

 

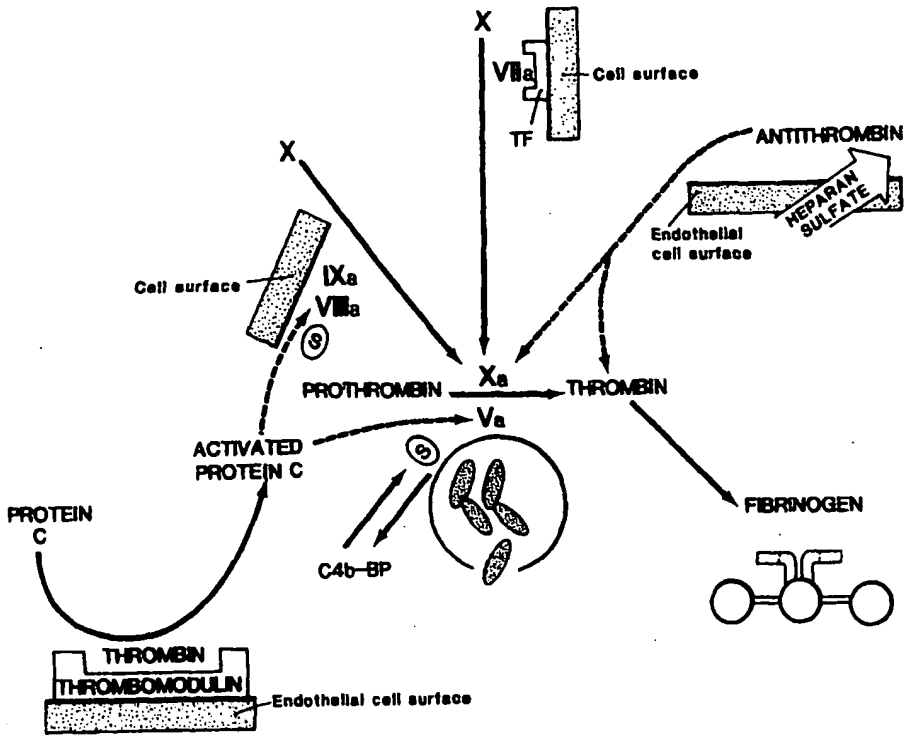


**Figure 1. The pathways that generate factor Xa and thrombin and the natural anticoagulant mechanisms that regulate activity of these enzymes.** After generation, factor Xa binds to factor Va on activated platelets, mediating conversion of prothrombin to thrombin; the latter, in turn, acts on fibrinogen to form a fibrin clot. Activated protein C functions as a potent anticoagulant by inactivating factors VIIIa and Va. Factor V Leiden mutation results in a form of coagulation factor V that, when activated to factor Va, is relatively resistant to degradation by activated protein C. This defect, known as resistance to activated protein C, is the most common inherited predisposition to thrombosis currently known. Reproduced with permission from Millenson MM, Bauer KA. Pathogenesis of venous thromboembolism. In: Hull R, Pineo GF, eds. Disorders of Thrombosis. Philadelphia: WB Saunders; 1996:175-90.

## Methods

English-language articles published between 1993 and April 1997 that addressed resistance to activated protein C or the factor V Leiden mutation with regard to laboratory diagnosis, prevalence, risks for thromboembolic disease, screening, and management were identified by a search of the MEDLINE database. Search terms included *factor V*, *mutation*, *protein C*, *resistance*, *thromboembolism*, *prevalence*, *diagnosis*, *screening*, *therapy*, and *prevention*. We reviewed the articles obtained from this search and additional articles identified from a review of the bibliographies. Data from case–control and prospective cohort studies were chosen for inclusion. In clinical areas where analytic data were lacking, illustrative case reports or case series were also chosen for inclusion.

## Data Synthesis

### Diagnosis of Resistance to Activated Protein C and Factor V Leiden Mutation

Although a definitive genetic diagnosis of factor V Leiden mutation status can be made by using techniques based on polymerase chain reaction (9–12), relatively simple plasma testing for resistance to activated protein C is available. The plasma-based test is performed by measuring the activated partial thromboplastin time in the presence and absence of activated protein C; results are expressed as a ratio of these values, normalized to pooled plasma (5, 32). A reduced ratio correlates well with heterozygosity or homozygosity for factor V Leiden mutation (**Figure 2**).

Many factors influence these values, and standard assays for resistance to activated protein C are less reliable when a patient is receiving anticoagulant therapy (32–35) or has lupus anticoagulant (35). Protein S deficiency does not appreciably alter results (34). Assay methods that use factor V–deficient plasma overcome these limitations and are now available as commercial kits that can be performed in most laboratories (33–37). Genetic confirmation for the presence or absence of factor V Leiden mutation is generally recommended for persons with a positive result on plasma-based testing for resistance to activated protein C.

### Prevalence

Unlike previously described inherited defects of hemostasis, factor V Leiden mutation is common. The carrier frequency of factor V Leiden mutation in healthy control populations ranges from 3% to

7% in Europe and the United States and may be as high as 15% in some selected groups (7, 15, 38, 39).

In the largest population study to date, the prevalence of factor V Leiden mutation was determined in a cohort of 4047 U.S. men and women (39). In this cross-sectional study, the overall carrier frequency for the mutation was 3.71% (95% CI, 3.14% to 4.33%) and the allele frequency was 1.89% (CI, 1.61% to 2.21%). The observed distribution of genotypes was consistent with that predicted by the Hardy–Weinberg equilibrium. Prevalence of factor V Leiden mutation was highest in white persons (5.27% [CI, 4.42% to 6.22%]) and was significantly less prevalent in other ethnic groups (2.21% in Hispanic Americans, 1.23% in African Americans, 0.45% in Asian Americans, and 1.25% in Native Americans) ($P < 0.001$) (39). Other reports also show that the mutation is not distributed equally among ethnic groups (38, 40–42): It is notably uncommon in Asian and African populations, a fact that may explain the decreased risk for venous thromboembolism in these groups (43, 44).

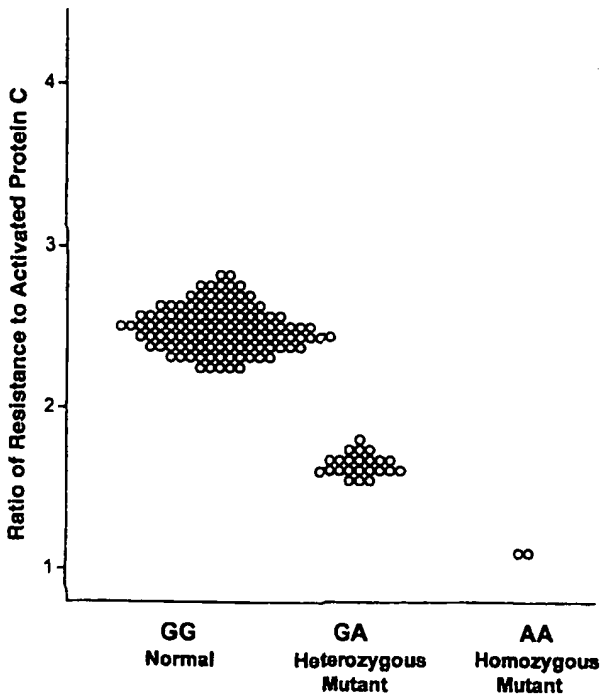


**Figure 2. Relation between ratio of resistance to activated protein C and carrier state for factor V Leiden mutation.** A = adenine; G = guanine.

### Factor V Leiden Mutation and Risk for Venous Thromboembolism

The relation between factor V Leiden mutation and venous thromboembolism was first recognized in families severely affected by recurrent thrombosis (5, 6). Of 14 families with thrombophilia, 9 (64%) had resistance to activated protein C; the disorder was inherited in a manner consistent with an autosomal dominant trait (5, 6). Affected persons in these families have reduced thrombosis-free survival (**Figure 3**) (13). In another selected series of unexplained juvenile or recurrent thrombosis, the prevalence of resistance to activated protein C was 52% to 64% (45). Familial thrombosis and juvenile thrombosis are relatively rare, and it was initially uncertain whether factor V Leiden mutation was also an important risk factor for venous thrombosis in the general population.

The significance of resistance to activated protein C as a risk factor for venous thromboembolism in the general population was shown in the Leiden Thrombophilia Study, a population-based case–control study of 301 patients younger than 70 years of age who had a first episode of deep venous thrombosis not related to a malignant condition (7). Resistance to activated protein C was detected in 21% of patients with deep venous thrombosis compared with 5% of age- and sex-matched controls; this yielded an almost sevenfold (matched odds ratio, 6.6 [CI, 3.6 to 12.0]) increase in risk for deep venous thrombosis in persons with resistance to activated protein C. Subsequent analysis showed that 80% of these persons with resistance to activated protein C were heterozygous or homozygous for factor V Leiden mutation (9). Other studies support the high correlation between resistance to activated protein C and factor V Leiden mutation (10–13). In referral populations of patients with venous thromboembolism, factor V Leiden mutation is the most common identifiable risk factor (prevalence, 11% to 37%) (6, 7, 12, 14, 15).

The association between factor V Leiden mutation and the risk for venous thromboembolism was confirmed in a large prospective study of apparently healthy men participating in the Physicians' Health Study (15). Among 14 916 men who had no history

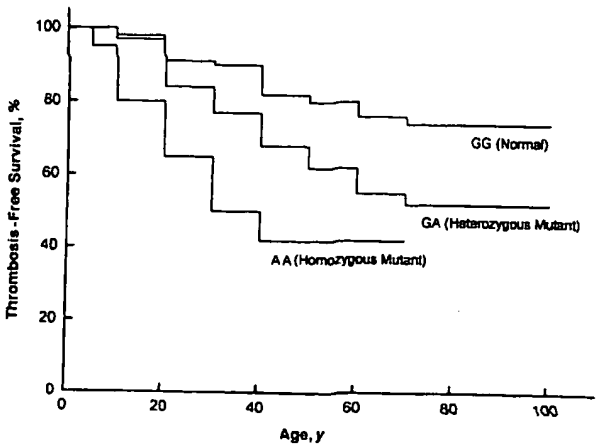


**Figure 3. Thrombosis-free survival in persons with familial thrombophilia.** A = adenine; G = guanine. Adapted from Zoller B, Svensson PJ, He X, Dahlbäck B. Identification of the same factor V gene mutation in 47 out of 50 thrombosis-prone families with inherited resistance to activated protein C. J Clin Invest. 1994;94:2521-4.

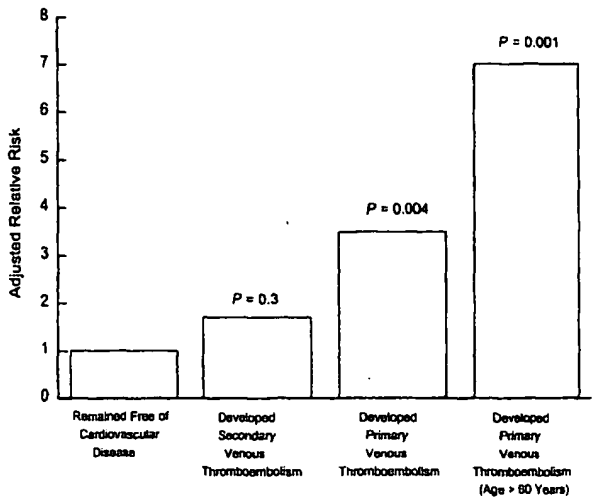


**Figure 4. Adjusted relative risks for future venous thromboembolism among apparently healthy men with factor V Leiden mutation.** Secondary venous thromboembolism includes events associated with cancer, surgery, or trauma. Adapted from Ridker PM, Hennekens CH, Lindpaintner K, Stampfer MJ, Eisenberg PR, Miletich JP. Mutation in the gene coding for coagulation factor V and the risk of myocardial infarction, stroke, and venous thrombosis in apparently healthy men. N Engl J Med. 1995;332:912-7.

of cardiovascular disease or cancer, 121 cases of venous thromboembolism accrued during a mean follow-up period of 8.6 years. The prevalence of the mutation was 11.6% among men with venous thromboembolism compared with 6.0% among those who remained healthy during follow-up (relative risk, 2.7 [CI, 1.3 to 5.6]; $P = 0.008$). In patients whose events were considered idiopathic, the risk imparted by the mutation was higher (relative risk, 3.5 [CI, 1.5 to 8.4]; $P = 0.004$). No association was seen between factor V Leiden mutation and thromboembolism associated with surgery, trauma, or cancer (secondary events) (relative risk, 1.7 [CI, 0.6 to 5.3]; $P = 0.3$). The effects of the mutation seemed greatest among men older than 60 years of age; in this subgroup, the adjusted relative risk was 4.0 (CI, 1.6 to 9.7; $P = 0.003$) for any type of venous thrombosis and 7.0 (CI, 2.6 to 19.1; $P < 0.001$) for primary venous thrombosis (**Figure 4**).

Patients homozygous for factor V Leiden mutation seem to be at even higher risk. In the Leiden Thrombophilia Study, the risk for venous thromboembolism among patients homozygous for factor V Leiden mutation was increased 80-fold (CI, 22-fold to 289-fold) (7, 14). Furthermore, homozygous patients who were presented with initial thromboembolism at a younger age (median age, 31 years) than did those who were heterozygous (median age, 44 years) or unaffected (median age, 46 years) by the mutation (14).

In cross-sectional and retrospective studies, the age at first thromboembolic event has been reported to be slightly less in patients heterozygous for factor V Leiden mutation than in persons without the mutation (5, 7, 9, 14, 45). However, reports of increased thromboembolic risk among young carriers of factor V Leiden mutation probably reflect an increased prevalence of trauma, use of oral contraceptives, and pregnancy in this group compared with older persons. In studies not confounded by the presence of these acquired risk factors, risks associated with factor V Leiden mutation increase with age. In the Physicians' Health Study, for example, the incidence of thromboembolism among heterozygous male carriers of factor V Leiden mutation increased with age at a rate significantly greater than that in unaffected men ($P$ for trend across age groups = 0.008) (46). In particular, for men 70 years of age or older, the incidence of venous thromboembolism in genetically affected men was significantly greater than that in unaffected men (7.83 compared with 1.86 events per 1000 person-years; $P = 0.028$) (46).

### Interaction with Concomitant Inherited Defects of Hemostasis

Factor V Leiden mutation enhances the risk for thrombosis in patients with other thrombophilic states, such as protein C and protein S deficiencies or hyperhomocystinemia (24–29). In a study of 113 patients with symptomatic protein C deficiency, the prevalence of the factor V Leiden mutation was 14% (24). In a study of seven families with both protein S deficiency and factor V Leiden mutation, 72% of members with both defects had a thrombotic event compared with 19% of those with only protein S deficiency and 19% of those with only factor V Leiden mutation (26).

Factor V Leiden mutation also seems to enhance the thrombotic risk associated with both inherited and acquired forms of hyperhomocystinemia (28, 29). In one study of 11 patients with homozygous familial homocystinuria, the 6 who demonstrated clinically apparent thrombosis were heterozygous or homozygous for factor V Leiden mutation (28). Factor V Leiden also seems to modulate the thrombotic risk associated with moderately elevated homocysteine levels because of less severe genetic defects or inadequate intake of vitamin $B_6$, vitamin $B_{12}$, or folate. In a prospective study of men evaluated for moderate hyperhomocystinemia and factor V Leiden mutation, those with both disorders had a 10-fold increase in risk for any form of venous thromboembolism (relative risk, 9.65; $P = 0.009$) and a 20-fold increase in the risk for idiopathic venous thromboembolism (relative risk, 21.8; $P = 0.0004$) compared with men who had neither abnormality (**Figure 5**, *left*). In this study, the risk for developing idiopathic thromboembolism in doubly affected persons was significantly greater than the risk associated with either condition alone (29); this observation may help explain why some (47–50)

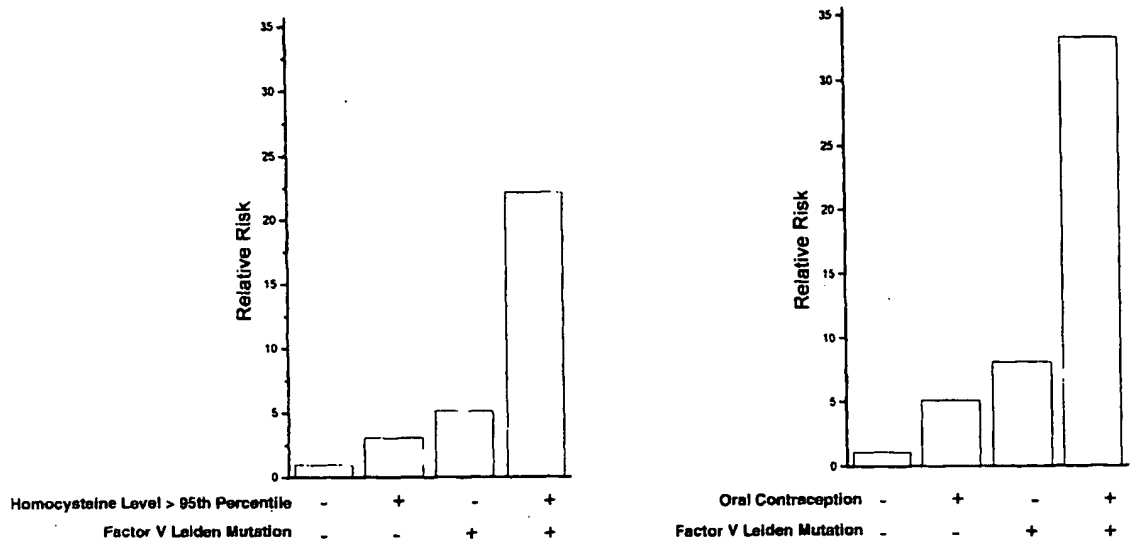


**Figure 5. Interrelations of factor V Leiden mutation and 1) hyperhomocystinemia (*left*) and 2) oral contraceptive use (*right*) on risks for venous thromboembolism.** + = present; − = absent. Adapted from Ridker PM, Hennekens CH, Selhub J, Miletich JP, Malinow MR, Stampfer MJ. Interrelation of hyperhomocyst(e)inemia, factor V Leiden, and risks of future venous thromboembolism. Circulation. 1997;95:1777-82; and from Vandenbroucke JP, Koster T, Briet E, Reitsma PH, Bertina RM, Rosendaal FR. Increased risk of venous thrombosis in oral-contraceptive users who are carriers of factor V Leiden mutation. Lancet. 1994;344:1453-7.

but not all (51, 52) previous studies of hyperhomocystinemia found positive associations with venous thromboembolism.

### Use of Oral Contraceptives and Postmenopausal Estrogen Replacement

Oral contraceptives increase the risk for venous thromboembolism (53). A recent World Health Organization case–control study of 1143 women with venous thromboembolism (54) showed that the relative risk among persons using oral contraceptives compared with persons who did not use oral contraceptives was 4.15 (CI, 3.09 to 5.57) in Europe and 3.25 (CI, 2.59 to 4.08) in developing countries.

As with other inherited thrombophilic states (55), use of oral contraceptives seems to augment the increased risk for venous thromboembolism associated with factor V Leiden mutation (18–21). In a comparison between 155 consecutive premenopausal woman who presented with deep venous thrombosis not related to a malignant condition and a control sample of 169 premenopausal woman without history of venous thromboembolism, the risk for thromboembolic events was increased fourfold with oral contraceptive use alone (relative risk, 3.8 [CI, 2.4 to 6.0]) and increased eightfold with factor V Leiden mutation alone (relative risk, 7.9 [CI, 3.2 to 19.4]). However, the risk for thromboembolic events was increased more than 30-fold in women with factor V Leiden mutation who also used oral contraceptives (relative risk, 34.7 [CI, 7.8 to 154]) (**Figure 5**, *right*) (18).

Oral contraceptives with a third-generation progestogen result in a significantly greater risk for venous thromboembolism both in persons who carry factor V Leiden mutation and normal persons than in persons who used contraceptives that contained a second-generation progestogen (19, 56). This observation suggests that estrogen content may not be solely responsible for the thrombotic risk seen in users of oral contraceptives.

Increased risks for thromboembolism associated with oral contraceptive use and factor V Leiden mutation are supported by other, smaller studies (19–21). Persons who are homozygous for factor V Leiden may be at especially high risk in this setting (20). It has also been suggested that oral contraception may cause resistance to activated protein C independent of the factor V mutation (57–59). The clinical effect of acquired resistance to activated protein C is uncertain.

The use of estrogen as hormone replacement therapy in postmenopausal women has not traditionally been considered a major risk factor for venous thromboembolism (60–63). However, recent studies have challenged this view (64–66). Specifically, two case–control studies from the United Kingdom (64) and the United States (65) showed that the relative risk for idiopathic thromboembolism was approximately 3.5 in users of oral estrogen replacement compared with nonusers (relative risk in the British study, 3.5 [CI, 1.8 to 7.0]; relative risk in the U.S. study, 3.6 [CI, 1.6 to 7.8]). In addition, a large prospective cohort study (66) demonstrated that in 123 cases of primary pulmonary embolism, the risk was significantly higher in current users of postmenopausal hormones than in nonusers (adjusted relative risk, 2.1 [CI, 1.2 to 3.8]).

No studies have examined the potential interaction between factor V Leiden mutation and hor-

mone replacement therapy. However, compared with women using oral contraceptives, those receiving replacement therapy tend to be older and thus have a higher absolute risk for idiopathic thromboembolism. Thus, the absolute increase in risk associated with factor V Leiden mutation may be greater among persons using hormone replacement therapy than among those using oral contraceptives.

### Risk for Venous Thromboembolism during Pregnancy

Pregnancy and the puerperium predispose women to venous thromboembolism, which is a major cause of maternal death (67, 68). The cause of the increased risk is uncertain but may be related to pregnancy-induced changes in hemostasis (69–71). In this regard, associations between venous thromboembolism during pregnancy and factor V Leiden mutation have been reported (21–23). In small case series, 40% to 59% of women with pregnancy-related venous thromboembolism had resistance to activated protein C or factor V Leiden mutation. In one study (22), carriers of the mutation seemed more prone to develop thrombosis during the first pregnancy. In another study of 50 patients with second-trimester pregnancy loss (72), the prevalence of resistance to activated protein C was 20%, significantly higher than the prevalence in women who only had a history of first-trimester miscarriage (5.7%; $P < 0.02$) and the prevalence in controls (4.3%; $P < 0.02$). Finally, studies have shown that resistance to activated protein C can be acquired during pregnancy (23, 73). All of these observations require confirmation in larger controlled studies.

### Risk for Recurrent Venous Thromboembolism

An important clinical problem for patients with a first episode of thromboembolism is the high risk for recurrence. In a recent long-term follow-up study of 355 patients with a first episode of symptomatic venous thrombosis, the incidence of recurrent disease was 30.3% after 8 years (CI, 23.6% to 37.0%) (74). The presence of factor V Leiden mutation may be associated with an increased rate of recurrence (16, 17). One prospective study followed 77 men who survived an initial episode of idiopathic venous thromboembolism for an average of 68.3 months (16). Persons with factor V Leiden mutation were four times more likely to have a recurrent event (relative risk, 4.1; $P = 0.04$), such that 76% of recurrences were attributable to mutation. All recurrences occurred after cessation of standard anticoagulant therapy.

Another prospective trial of 251 patients with a first episode of deep venous thrombosis reported that 16.3% carried factor V Leiden mutation (17). All patients were followed for as long as 8 years (mean duration of follow-up, 3.9 years), during which time those with factor V Leiden mutation had a higher risk for recurrent thromboembolism than did those without the mutation (39.7% compared with 18.3%; hazard ratio, 2.4 [CI, 1.3 to 4.5]; $P < 0.01$).

A smaller retrospective study compared 21 patients who had factor V Leiden mutation (5 homozygotes and 16 heterozygotes) with age- and sex-matched controls who had venous thromboembolism without mutation (75). All 5 homozygotes had had recurrent events before the study period compared with 9 of 16 heterozygotes and 9 of 21 persons without mutation. During the observation period, homozygotes had a rate of recurrence of 9.5% per patient per year. Patients who were heterozygous for the mutation did not have a significantly higher rate of recurrence than did those without the mutation (4.8% per patient per year compared with 5% per patient per year); follow-up in this study was limited.

### Risk for Arterial Thromboembolism

Arterial thromboembolism, predominantly myocardial infarction and stroke, is the leading cause of illness and death in the United States. After two case reports of early myocardial infarction in patients homozygous for factor V Leiden mutation (76) and other case reports suggesting an association with stroke (77–79), several investigators hypothesized that this mutation might be an important risk factor for arterial thrombotic events. In the largest study to address this issue, however, no significant difference in the prevalence of the factor V mutation was found between men with myocardial infarction (6.1%; $P > 0.2$) or cerebrovascular disease (4.3% percent; $P > 0.2$) and men without cardiovascular disease (6%) (15). Furthermore, most population-based and case–control studies confirm that factor V Leiden mutation is not a risk factor for myocardial infarction (80–83) or cerebrovascular disease (84–86).

### Screening for Factor V Leiden Mutation

Venous thromboembolism is associated with more than 300 000 hospitalizations annually in the United States, and pulmonary embolism is directly responsible for 50 000 to 100 000 deaths each year (87–89). However, despite strong associations between factor V Leiden mutation and risks for thromboembolism, decisions on whether to screen for this inherited defect are complex and vary in different clinical settings.

For example, the lifetime risk for venous thromboembolism among unselected persons is lower than the population prevalence of the factor V Leiden mutation. Thus, screening programs for primary

prevention are likely to be inefficient. In fact, if persons found to carry the mutation are subsequently advised to use long-term anticoagulant therapy, screening intended for primary prevention could result in a net clinical hazard because the lifetime risks of anticoagulation are substantial.

In contrast, screening for factor V Leiden mutation may prove effective among patients who have had a first episode of venous thromboembolism and are at substantial risk for recurrence. In this group, however, the risks for recurrent thromboembolic disease associated with the factor V Leiden mutation seem to be limited to events without a short-term risk factor (16). Thus, it is unlikely that screening before surgery is warranted, particularly because adequate thromboprophylaxis in the postoperative period reduces risks in all patients regardless of factor V Leiden status (90). Moreover, no data from randomized trials are available to show that long-term prophylaxis will prevent recurrent thromboses among patients identified as carrying the factor V Leiden mutation. Clinical trials designed to evaluate the benefit-to-risk ratio of factor V Leiden mutation screening and subsequent long-term anticoagulation are therefore needed. Such trials will need to consider that differential rates of mutation occur in various patient groups (39, 40) and that thrombosis associated with factor V Leiden mutation is not limited to young patients (47).

Among pregnant women, maternal death from pulmonary embolism is rare, occurring in 1 to 5 of every 100 000 deliveries (68, 91). If half of these events occurred among the 5% of women who carry factor V Leiden mutation, then the frequency of puerperal death from pulmonary embolism among women with the mutation ranges from 1 in 2000 to 1 in 15 000 (92). However, it has also been estimated that the risks for severe complications associated with anticoagulation during and after pregnancy might also affect approximately 1 in 2000 women (92). Thus, in this higher-risk group, screening for factor V Leiden mutation also has uncertain utility.

Among young women using oral contraceptives, the risk for venous thrombosis is increased, particularly among those with factor V Leiden mutation (18, 20). In this age group, however, the absolute risk is sufficiently low that even large differences in relative risks may be of limited importance. For example, the estimated incidence of venous thromboembolism in young women is 2 per 10 000 person-years, and the incidence of fatal pulmonary embolism is 6 per 100 000 person-years (92). Assuming that all fatal events are attributable to factor V Leiden mutation, almost half a million women would require screening to identify the 20 000 to 25 000 women who carry the mutation and would be denied oral contraception in order to avoid one death per year (92). In this setting, the costs of such a program are formidable and the possibility of a net medical hazard must again be considered, particularly if women denied oral contraception do not obtain an alternative and equally effective form of birth control.

Thus, until clinical trials showing efficacy of long-term therapy are undertaken, screening for the factor V Leiden mutation is likely to remain clinically important for persons with a family or personal history of thrombophilia. In this group, knowledge of factor V Leiden mutation status may be particularly useful if the affected person or family member is found to be homozygous (14). It is important to recognize, however, that the identification of those who should avoid oral contraception and those who will require gestational or puerperal anticoagulation can often be achieved by a complete family and personal history in which previous thromboembolic events are carefully emphasized (93).

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To write prescriptions is easy, but to come to an understanding with people is hard.

Franz Kafka
*The Country Doctor*

Submitted by:
Roger E. Nieman, MD
Abington, PA 19001

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Evidence Appendix B

# The HR2 Haplotype of Factor V: Effects on Factor V Levels, Normalized Activated Protein C Sensitivity Ratios and the Risk of Venous Thrombosis

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## Key words

HR2 haplotype, factor V, APC resistance, venous thrombosis

## Summary

We studied the HR2 haplotype of the factor V gene in a case-control study for venous thrombosis including 474 patients with a first deep-vein thrombosis and 474 age- and sex-matched healthy controls (Leiden Thrombophilia Study, LETS). We investigated both the original His1299Arg (A4070G) polymorphism and the Met385Thr (T1328C) polymorphism. This latter polymorphism, located in exon 8 (heavy chain), is always present in the HR2 haplotype, but also occurs on its own in a His1299 (wt) background. The HR2 haplotype was not associated with an increased risk of venous thrombosis (OR = 1.2, 95% confidence interval: 0.8-2.0). We did not find an association between the HR2 haplotype and a reduced sensitivity for activated protein C (APC) in non-carriers of factor V Leiden (FVL). However, in compound heterozygous FVL/HR2 carriers the sensitivity for APC was reduced. The HR2 haplotype was also associated with reduced factor V antigen levels in both patients and controls. Sequence analysis of the promoter region of factor V in HR2 homozygotes did not reveal any sequence variations that could explain the reduced FV levels. Our results show that the HR2 haplotype is not associated with an increased risk of venous thrombosis or with a reduced sensitivity for APC in non-FVL carriers. However, the HR2 haplotype is associated with a reduced sensitivity for APC in carriers of FVL and with reduced factor V antigen levels.

## Introduction

Human coagulation factor V (FV), which is synthesized in the liver and in megakaryocytes, circulates in plasma as a 330 kD single chain glycoprotein. The domain organization (A1-A2-B-A3-C1-C2) of FV is similar to that of factor VIII (FVIII) (1). By selective proteolytic cleavages the large B-domain is removed, yielding activated FV (FVa) which consists of a heavy chain (A1-A2) and a light chain (A3-C1-C2) that are noncovalently linked by a calcium ion [for a review see Rosing and Tans (2)]. The activated FV molecule acts as a cofactor to activated factor X (FXa) in the prothrombinase complex that proteolytically activates prothrombin to thrombin (3). FVa is inactivated by activated protein C (APC) by selected proteolytic cleavages in the heavy chain (4). This inactivation, with protein S as cofactor, is an important step in the anticoagulant pathway. Activated FVIII (FVIIIa) is also inactivated by APC and FV is thought to function as a cofactor, synergistic with protein S, in this reaction (5-7).

Activated protein C resistance, a poor anticoagulant response of plasma to APC, is almost always associated with the presence of a mutation in one of the APC cleavage sites (Arg506) of FV (8, 9). The activated FV variant (factor V Leiden, FVL) is inactivated more slowly than activated wildtype FV (10-13). APC resistance caused by the FVL mutation is a common and strong risk factor for venous thrombosis (14, 15). Recently, we reported that a reduced sensitivity for APC not due to FV Leiden is also associated with an increased risk of venous thrombosis (16).

The gene for human FV is localized on chromosome 1q23-24 and consists of 25 exons and 24 introns (17). The B-domain is fully encoded by the large exon 13. In 1996, the His1299Arg [A4070G, according to the cDNA sequence of Jenny et al. (1)] polymorphism in exon 13 was first described (18). In this study, the Arg1299 (R2) allele was reported to be more frequent in subjects with reduced FV activity levels. Subsequent studies which have investigated the HR2 haplotype have diverse results. Bernardi et al. reported that the R2 allele was associated with a reduced sensitivity for APC (19). An association with reduced FV levels was not found in this Italian study, nor an indication that the R2 allele is a risk factor for venous thrombosis. A French case-control study did show relationships between the HR2 haplotype and reduced FV levels and a reduced response to APC (20). Besides, this study showed that the R2 allele is associated with a 1.8-fold increased risk of venous thromboembolism. One family study showed that compound heterozygous FVL/HR2 carriers have a more reduced normalized APC-SR than FVL heterozygotes (21). The HR2 haplotype includes, in addition to the R2 allele, 7 other polymorphisms in exon 13 and one in exon 16. Four of these variations do not cause aminoacid substitutions and most of these polymorphisms have a much higher population frequency than the His1299Arg variation. The HR2 haplotype has an allele frequency of 8% in Italians and of 6% in the French study (19, 20).

We studied the HR2 haplotype in a population-based case-control study on venous thrombosis (Leiden Thrombophilia Study, LETS). We screened for the His1299Arg polymorphism as well as for a novel variation, the Met385Thr polymorphism. This latter polymorphism, which was detected by sequencing of the FV gene of a FVL/HR2 compound heterozygote, is also part of the HR2 haplotype and is located in the

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heavy chain of the FV molecule. Furthermore we investigated the association between the HR2 haplotype and FV antigen levels and the sensitivity for APC. We also assessed the risk of venous thrombosis in compound heterozygous carriers of the R2 allele and the FVL allele.

## Patients and Methods

*Subjects*

The Leiden Thrombophilia Study (LETS) is a population-based case-control study in three Dutch anticoagulation clinics. The study, of which the design has been described extensively elsewhere (22), includes 474 patients with a first episode of deep venous thrombosis and 474 age- and sex-matched healthy control subjects. Venepuncture took place at least 6 months after the thrombotic event. DNA analysis could be performed for 943 individuals (471 patients and 472 controls). For these individuals the ratio of male to female subjects was 1.3 for both patients and controls. The mean age was 47 years for both groups (range 16-70 for the patients and 16-73 for the controls).

*Blood Collection and Laboratory Analysis*

Blood was collected into tubes containing 0.106 mol/L trisodium citrate. Plasma was prepared by centrifugation for 10 min at 2000 g at room temperature and stored at –70° C. The sensitivity of the plasma activated partial thromboplastin time (APTT) to APC was measured as described before (22). Results were expressed as normalized APC sensitivity ratios (n-APC-SR). The APC sensitivity ratio is defined as the APTT in the presence of APC divided by the APTT in the absence of APC. The normalized APC-SR is calculated by dividing the APC-SR of the sample by the APC-SR of pooled normal plasma which is measured in the same run.

FV antigen (ag) was measured by a sandwich type enzyme-linked immunosorbent assay (ELISA) using two different monoclonal antibodies (V-6, V-9) against the light chain of FV (23). Briefly, wells coated with monoclonal antibody V-6 were incubated with diluted plasma sample. Monoclonal antibody V-9, conjugated to horseradish peroxidase, was used for the detection of immobilized FV. FVag levels were expressed in units per deciliter (U/dl). By definition 1 ml pooled normal plasma contains 1 unit.

FVIIIag levels were measured by a sandwich type ELISA with two different monoclonal antibodies directed against the light chain of FVIII (Kamphuisen et al., submitted). Briefly, wells coated with monoclonal antibody CLB Cag 117 were incubated with a diluted plasma sample. Monoclonal antibody CLB-Cag A conjugated to horseradish peroxidase was used for detection. Monoclonal anti-FVIII antibodies were kindly provided by Dr J. van Mourik (CLB, Sanguin Blood Supply Foundation, Amsterdam, The Netherlands).

FVIII coagulant activity (FVIII:C) was measured by a one-stage clotting assay as described before (24). Protein C activity and antithrombin activity were measured with Coamate (Chromogenix, Mölndal, Sweden) on an ACL-200 (Instrumentation Laboratory, Milan, Italy), factor II activity with a chromogenic method using S-2238 (Chromogenix) and Echis carinatus snake venom (Sigma Chemical Co, St Louis, USA) on an ACL-200 (25), and factor X antigen was measured by ELISA with a polyclonal antibody (DAKO. Denmark) (de Visser et al., in preparation). Factor VII was measured using Thromborel S reagent (Behringwerke AG, Warburg, Germany) and factor VII deficient plasma (Organon Teknica, Durham, USA) (26). Total protein S was measured by polyclonal ELISA (27) and free protein S was measured directly in plasma by ELISA using two monoclonal antibodies specific for free protein S (Asserachrom free protein S, Diagnostica Stago, Asnieres-sur-Seine, France) (28, 29). The fibrinogen concentration was determined according to method of Clauss using Dade® thrombin reagent (Baxter, Miami, USA) on an Electra 1000 (MLA, Pleasantville, USA) (26).

The results of all the above mentioned measurements, except for FVag, FVIIIag and FXag have been reported previously (22, 24, 26, 30).

High molecular weight DNA was isolated from leukocytes and stored at 4° C. A list of the primers used for sequencing of all exons of FV in the FVL/HR2 heterozygote and a description of the sequencing procedure have been reported elsewhere (31). The detection of three polymorphisms (A4070G, T1328C and A6755G; numbering according to Jenny et al. (1)) in the FV gene was performed by polymerase chain reaction (PCR) followed by restriction enzyme digestion. The PCR mixture consisted of 50 ng of both oligonucleotides, 200 μM of each dNTP, 67 mM Tris-HCl pH 8.8, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 6.7 μM EDTA, 16.6 mM $(NH_4)_2SO_4$, 0.5 mg/ml BSA, 0.2 units AmpliTaq polymerase (Perkin-Elmer) and 10% DMSO (only for the A4070G and A6755G polymorphisms) in a total volume of 10 μl. The reactions were performed in a T3 Thermocycler (Biometra, Göttingen, Germany). The PCR conditions were as follows: 4 min initial denaturation at 94° C, followed by 33 cycles of 1 min at 94° C, 1 min at 65° C and 90 sec at 72° C. A final extension was performed at 72° C for 4 min. For detection of the A4070G (= His1299Arg) polymorphism a 828 bp fragment was amplified with primer A (5'-CATGAAGTCTGGCAGACAGTC-3') and primer B (5'-TATCTGGCTGAGATCCGGGAG-3'). The T1328C (= Met385Thr) polymorphism was determined after amplification of a 153 bp fragment with primer C (5'-CACCAAACATACAGTGAATCCCAGTA-3') and primer D (5'-AATAACCAGGTACTCCATAATATTTTAC-3'). The underlined nucleotide in primer C corresponds to a mismatch with the gene sequence and was introduced to create a RsaI restriction site (in the presence of the 1328C allele) for detection of the polymorphism. For detection of the Asp2194Gly (= A6755G) polymorphism a 440 bp fragment was amplified with primers E (5'-GTGTTCTATGTGTTCTTTGATATCCTCATT-3') and F (5'-GGGTTTTTGAATGTTCAATTCTAGTAGATA-3'). PCR products were digested by incubation with either RsaI (New England Biolabs, A4070G and T1328C polymorphisms) or with EcoRV (New England Biolabs, A6755G polymorphism) overnight at 37° C. The restriction fragments (A4070G polymorphism: 828 bp for the 4070A allele, and 381 and 447 bp for the 4070G allele; T1328C polymorphism: 142 and 11 bp for the 1328T allele, and 117, 25 and 11 bp for the 1328C allele; A6755G polymorphism: 390, 29 and 21 bp for the 6755A allele, and 419 and 21 bp for the 6755G allele) were separated in 2% agarose gels and visualized after ethidium bromide staining. The determination of the FVL mutation in the LETS samples has been described previously (9). Almost 2 kb of the upstream region of the FV gene (bases -1 to -1933, according to GenBank sequence U83346) was amplified by PCR with primers G (5'-TCAGTAGGCTAGGTGTTCTAGGAC-3') and H (5'-GCTTCCTTTCCTGCTCCCGC-3') with the Expand Long Template PCR System (Boehringer Mannheim). Sequencing of this PCR fragment was performed with the ABI Prism® BigDye terminator cycle sequencing ready reaction kit (Perkin-Elmer Applied Biosystems) according to manufacturer's protocol. Sequences of the used primers can be asked for. The reactions were run on an ABI Prism 310 (Perkin-Elmer Applied Biosystems).

*Statistical Analysis*

Odds ratios (OR) were calculated in the standard unmatched fashion. Ninety-five percent confidence intervals (95% CI) were constructed according to Woolf (32). The OR is used as an estimate of the relative risk, which indicates the risk of developing venous thrombosis in a category of exposure (e.g., HR2 carriers) relative to the reference category (e.g., HR2 wildtype). An OR of 1 indicates no effect on risk, while an OR above 1 indicates an increase in risk.

## Results

*The Met385Thr Polymorphism*

In the population-based case-control study LETS, one heterozygous FVL carrier was present with a normalized APC-SR of 0.44. In our laboratory this ratio is within the range of homozygous FVL carriers (n-APC-SR <0.45) (33). Because of this discrepancy all exons of the FV gene were sequenced in this individual. Besides the FVL mutation and some previously described polymorphisms, the A4070G (His1299Arg) variation, which is part of the HR2 haplotype, was detected in heterozygous form (19). Previous investigations had already shown that the FVL allele is not present in the HR2 haplotype (34-37).

Furthermore one novel variation (T1328C, Met385Thr) in exon 8 was detected. This latter variation was not present in 8 homozygous FVL carriers (Guasch et al., unpublished results), so the Thr385 allele is not part of the FVL haplotype. Screening of a panel of 90 normal individuals for the His1299Arg and Met385Thr variations revealed 9 individuals who were heterozygous for both variations. Two individuals were homozygous for His1299 and heterozygous for Met385Thr. So, the Thr385 allele is always present in the HR2 haplotype, but can also occcur on its own in a His1299 (wildtype, R1) background. Previous studies (19, 20) have reported an association between reduced normalized APC-SR and the presence of the HR2 haplotype, but no plausible explanation for this reduction has been found yet. The Met385Thr polymorphism is located in the heavy chain of FV, which is directly involved in the generation of thrombin and the inactivation of FVa by APC. Therefore we hypothesized that this variation might be responsible for the reduced normalized APC-SR. Because the two variations His1299Arg and Met385Thr are not in absolute linkage disequilibrium, we looked at both polymorphisms in LETS.

*Table 1* Frequencies of the His1299Arg and Met385Thr polymorphisms

| | | His1299Arg | | |
|---|---|---|---|---|
| | Met385Thr | ++ | +- | -- |
| Patients (n=471) | ++ | 2 | 0 | 0 |
| | +- | 0 | 46 | 8 |
| | -- | 0 | 0 | 415 |
| Controls (n=472) | ++ | 0 | 0 | 0 |
| | +- | 0 | 39 | 9 |
| | -- | 0 | 0 | 424 |

*Table 2* Co-inheritance of factor V Leiden and HR2 haplotype and the risk of venous thrombosis

| FVL | His1299Arg | Patients (n=461) | Controls (n=472) | OR | 95% CI |
|---|---|---|---|---|---|
| - | - | 340 | 420 | 1* | |
| + | - | 75 | 13 | 7.1 | 3.9–13.1 |
| - | + | 37 | 38 | 1.2 | 0.7–1.9 |
| + | + | 9 | 1 | 11.1 | 1.4–88 |

* Reference category

Subjects homozygous for factor V Leiden (8 patients) or homozygous for the R2 allele (2 patients) are not included in this table

*Table 3* Mean normalized APC sensitivity ratio according to factor V Leiden and the factor V His1299Arg polymorphism

| | | Patients | | Controls | |
|---|---|---|---|---|---|
| FVL | His1299Arg | n | n-APC-SR (95% CI) | n | n-APC-SR (95% CI) |
| ++ | -- | 8 | 0.43 (0.42 - 0.44) | 0 | |
| +- | -- | 63 | 0.57 (0.56 - 0.58) | 13 | 0.57 (0.56 - 0.59) |
| | +- | 8 | 0.51 (0.48 - 0.54) | 1 | 0.52 |
| -- | -- | 303 | 0.96 (0.95 - 0.97) | 415 | 1.02 (1.01 - 1.04) |
| | +- | 32 | 0.95 (0.92 - 0.99) | 38 | 1.03 (0.98 - 1.08) |
| | ++ | 2 | 0.92, 0.93 | 0 | |

Patients using oral anticoagulants or with a lupus anticoagulant were excluded for this analysis

*HR2 Haplotype and the Risk of Venous Thrombosis*

All subjects were screened for both the His1299Arg (A4070G) polymorphism in the B-domain and the Met385Thr (T1328C) polymorphism in the heavy chain of FV (Table 1). It was found that 2 of the 471 patients were homozygous for the R2 allele and 46 were heterozygous (allele frequency 5.3%). Thirty-nine of the 472 controls were heterozygous for the R2 allele and none of the controls was homozygous for the R2 allele (allele frequency 4.1%). The odds ratio (OR), calculated as a measure of the relative risk of venous thrombosis, for subjects carrying the R2 allele (in heterozygous or homozygous form) was 1.2 (95% CI: 0.8-2.0) compared to homozygous R1 (wildtype) carriers. In addition to all carriers of the R2 allele, seventeen homozygous carriers of the R1 allele (8 patients and 9 controls) were heterozygous for the Thr385 allele. The allele frequency for the Thr385 allele was 6.2% and 5.1% for patients and controls, respectively. The Thr385 allele was also not associated with an increased risk of venous thrombosis (OR = 1.2, 95% CI: 0.8-1.8).

*Compound Heterozygous HR2/Factor V Leiden Carriership and Risk of Venous Thrombosis*

We investigated whether co-inheritance of the R2 allele influenced the risk of venous thrombosis in heterozygous carriers of FVL (Table 2). Homozygous FVL or HR2 carriers were not included in this analysis because the FVL allele and the R2 allele are not present in the same haplotype (37). The OR for heterozygous carriers of FVL who are not carrying the R2 allele was 7.1 (95% CI: 3.9-13) compared to wildtype carriers (no HR2 and no FVL). The OR for compound heterozygous FVL/HR2 carriers was slightly higher (OR = 11, 95% CI: 1.4-88), but the confidence intervals largely overlapped.

*HR2 Haplotype and Normalized APC Sensitivity Ratio*

We investigated the relationship between the HR2 haplotype and the sensitivity for APC, which was measured in undiluted plasma by an APTT-based assay with Cephotest® as activator. The association between genotype and normalized APC-SR is shown in Table 3. All FVL homozygotes carried the R1 allele, because this allele is part of the FVL haplotype. Compound heterozygous carriers for FVL/R2 have reduced normalized APC-SRs compared to FVL/R1 carriers. In non-FVL carriers no difference in APC sensitivity was found between carriers of the R1 allele and carriers of the R2 allele in both patient and control groups. None of the 17 subjects who were only heterozygous for Met385Thr did carry the FVL mutation. For both patients and controls, no significant difference in mean normalized APC-SR was found between this group and non-FVL carriers who were homozygous for R1/Met385.

| | Patients | | Controls | |
|---|---|---|---|---|
| His1299Arg | n | Mean FVag (95% CI) | n | Mean FVag (95% CI) |
| -- | 423 | 136 (133 - 140) | 433 | 134 (131 - 137) |
| +- | 46 | 117 (107 - 128) | 39 | 107 (97 - 117) |
| ++ | 2 | 79, 105 | 0 | - |

*Table 4* Mean factor V antigen levels (U/dl) according to the factor V His1299Arg polymorphism

*HR2 Haplotype and Factor V Antigen Levels*

The association between the HR2 haplotype and FVag levels was investigated. The results are shown in Table 4. Mean FVag levels were 134 U/dl and 132 U/dl for patients and controls, respectively (23). The R2 allele was associated with reduced FVag levels in both patients and controls. To assess whether the reduction in FVag levels was specific and not due to a difference in liver function, mean levels of other coagulation factors were calculated for the different genotype subgroups. For the investigated coagulation factors (FVIIIag, FVIII:C, antithrombin, fibrinogen, factor II, factor VII, factor X, protein C, protein S [free and total]) no differences in mean levels were found between homozygous R1 carriers and heterozygous R1R2 carriers. To establish whether the reduction in FV levels was caused by the Thr385 variation we compared the mean FV levels of subjects homozygous for R1 and Met385 (N = 839, mean FVag = 135 U/dl, 95% CI: 133-137) and subjects homozygous for R1 but heterozygous for Met385Thr (N = 17, mean FVag = 124 U/dl, 95% CI: 108-141). For this analysis patients and controls were taken together because of the small size of the group of subjects heterozygous for the Met385Thr variant. Again, no difference in mean FV levels was found. So, the established reduction in FV levels in carriers of the HR2 haplotype is probably not due to the Thr385 allele.

*Promoter Polymorphisms*

We wondered whether the reduced FV levels that were found in carriers of the HR2 haplotype were caused by a polymorphism in the promoter region of FV in linkage disequilibrium with the R2 polymorphism. Therefore we sequenced 1933 basepairs of the promoter region of FV of the two HR2 homozygotes and one wildtype control. No sequence variations were identified between the three sequenced individuals. The sequences we found were identical to the PAC sequence submitted by Bird (GenBank accession number Z99572).

*His1254Arg Polymorphism*

During screening of the His1299Arg polymorphism, one aberrant *RsaI* restriction pattern was detected, suggesting the presence of the His1254Arg (A3935G) polymorphism in the FV gene which was previously described by Lunghi et al. (38). The presence of this variation in heterozygous form was confirmed by sequence analysis. Like the R2 polymorphism, the His1254Arg polymorphism is located in a highly repeated area of exon 13 with 31 tandem repeats of 27 bp. It is interesting that the two polymorphisms are located in exactly the same position (20th nucleotide) of two similar repeats (His1254Arg in the 11th repeat and His1299Arg in the 16th repeat). The female control carrying this variant had a normalized APC-SR of 0.78 and a FVag level of 117 U/dl, which is relatively low for the LETS population. She did not carry the R2 allele or the Thr385 allele.

*Asp2194Gly Polymorphism*

Recently, a missense polymorphism (A6755G) in exon 25 of the FV gene was reported, predicting an aminoacid substitution (Asp2194Gly) in the C2 domain of the FV molecule (39). This polymorphism was found to be tightly linked to the R2 allele. We screened all carriers of the R2 allele for this polymorphism and found that 2 of the 85 heterozygous carriers did not carry this novel variation. Two homozygous R2 carriers did carry the Gly2194 allele in homozygous form. Furthermore this variation in the light chain was not detected in 150 homozygous His1299 carriers. We had not detected this variation by sequence analysis of all FV exons of the compound heterozygous FVL/HR2 subject. Re-analysis revealed the presence of the Asp2194Gly polymorphism in heterozygous form. Our results confirm the tight linkage of this variation to the R2 allele.

## Discussion

The HR2 haplotype of the FV gene was not found to be associated with an increased risk in our population-based case-control study for venous thrombosis (LETS). These findings correspond with the results of Bernardi and Luddington (19, 40). Our results differ from the results of a recent French study that did find an increased risk of venous thrombosis associated with the HR2 haplotype (20). The study population of this French study was not exactly defined, but allele frequencies of FVL and the prothrombin G20210A variant in this French study were not different from the frequencies in our study (15, 20, 41).

Faioni et al. recently showed in a family study that co-inheritance of the R2 allele resulted in an increased risk of venous thromboembolism in FVL carriers (42). Our data do not support this observation, but cannot exclude it either. The OR for compound heterozygous FVL/HR2 carriers was slightly higher (OR = 11, 95% CI: 1.4-88) than the OR for heterozygous FVL carriers (OR = 7.1, 95% CI: 3.9-13), but the confidence intervals largely overlapped. Because the OR for compound heterozygotes depends on only one control, these data suffer from statistical uncertainty.

In addition to the His1299Arg polymorphism we studied the Met385Thr polymorphism in the heavy chain of FV. This variation, that had not been previously reported, is part of the HR2 haplotype and was detected by sequencing of a FVL heterozygote with an APC resistant phenotype similar to a homozygous FVL carrier. In our large population sample the Thr385 allele was always present in the HR2 haplotype, but it also occurred with the R1 allele. Just like the R2 allele, the Thr385 allele was not associated with an increased risk of venous thrombosis.

Recently, a missense polymorphism (A6755G) in exon 25 of the FV gene was reported, predicting an aminoacid substitution (Asp2194Gly) in the C2 domain of the FV molecule (39). We confirmed the tight linkage of this variation to the R2 allele by screening of all R2 carriers and 150 homozygous R1 carriers. It was reported that in carriers of the Gly2194 allele the ratio of the two isoforms of FV (FV1 and FV2) is shifted in favor of FV1, the isoform which has the highest overall procoagulant activity (39, 43, 44).

Our observation that the HR2 haplotype is associated with reduced FV levels corresponds with the results of all previous studies on the HR2 haplotype (18, 20, 21), except the study of Bernardi et al. (19). In this Italian study FV levels were only measured in a small sample of HR2 carriers and not in non-carriers. Our findings that the HR2 haplotype is associated with reduced FV levels but not with an increased risk of venous thrombosis agrees with the recent finding that reduced FV levels are not a risk factor for venous thrombosis (23). To assess whether the reduction in FV levels was due to the Thr385 allele we compared mean FV levels of subjects homozygous for R1 and Met385 and subjects homozygous for R1 but heterozygous for Met385Thr. No difference was found, so we showed that the Thr385 variation in the heavy chain is probably not responsible for the reduced FV levels in the HR2 haplotype. Another possible explanation for the reduced FV levels is that the His1299Arg polymorphism is in linkage disequilibrium with a polymorphism in the promoter region of the FV gene that may cause a reduced expression of the FV gene. To investigate this we sequenced a large upstream region (1933 bp) of two homozygous HR2 carriers and one wildtype control, but no sequence variations were detected. So, the mechanism by which the genotype is associated with reduced FV levels is not clear yet. The most simple explanation is that the His1299Arg variant itself is responsible for the reduction in FV levels. The substitution of histidine to arginine at amino acid 1299, which is located in a highly repeated region in exon 13 of the FV gene, will give a repeat with a unique sequence and could be responsible for reduced FV levels through the production of a less stable protein or interference with intracellular trafficking. One indication for this explanation is that the heterozygous carrier of the His1254Arg polymorphism, which is located in homologous position in the repeat as the His1299Arg mutation, also has a relatively reduced FV level (117 U/dl).

In our study population the HR2 haplotype was not associated with a reduced sensitivity for APC in non-FVL carriers. However, in compound heterozygous FVL/HR2 carriers, the normalized APC-SR was reduced compared to heterozygous FVL/R1 carriers. This observation corresponds with the results of the family study of Castaman et al. (21). It also corresponds with the results of previous reported plasma experiments in which a mixture of homozygous HR2 plasma and homozygous FVL plasma led to a reduced normalized APC-SR compared to FVL heterozygous plasma (19). Our finding that the HR2 haplotype in non-FVL carriers is not related with a reduced sensitivity for APC can be shared with a recent French study in which the APC ratio, measured with an APC resistance assay with undiluted plasma, in patients carrying the R2 allele was not different from patients not carrying the allele (20). In the control group of this latter study only a small difference in APC ratio, measured with a modified FV specific assay, was found. In the original study of Bernardi, who was the first to point to an association between the HR2 haplotype and a reduced sensitivity for APC, this relation was only proven in a small study population (19). The use of different APC resistance tests can also be the cause of the discrepancies between the above mentioned studies. The measured APC response depends on the type of clotting test and the particular reagent that is used. Besides, the influence of other factors (e.g. FVIII levels), apart from FVL, that determine the APC response may differ between assays (16, 45, 46). The observation that the effect of the HR2 haplotype on the normalized APC-SR is only found in FVL carriers can be explained by the fact that the APC resistance assay reflects, among other things, the ratio between the concentrations of the wildtype FV molecule and the FVL molecule in plasma (9). The R2 allele is associated with reduced FV levels and as a result the relative concentration of the FVL molecule increases, leading to a more reduced normalized APC-SR. More gravely, this effect is observed in compound heterozygous carriers of FVL and a quantitative FV deficiency (47-50). These so-called pseudo-homozygous FVL carriers have reduced normalized APC-SRs within the range of homozygous FVL carriers, due to the decrease in the relative concentration of the normal FV molecule.

We conclude that the HR2 haplotype is associated with reduced FVag levels and with a reduced sensitivity for APC in FVL carriers. It is not associated with a reduced sensitivity for APC in non-FVL carriers or with an increased risk of venous thrombosis. The mechanism which underlies the reduction of FV levels has to be further investigated.

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Evidence Appendix C

# Genetic Approach to Thrombophilia

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**Key words**

Thrombophilia, venous thrombosis, genetic risk factors, polymorphisms

**Summary**

Venous thrombosis is a multifactorial disease. Multiple interactions between genetic and environmental factors contribute to the development of the disease. Presently, we know of six or seven genetic risk factors for venous thrombosis. However, together these defects can explain the clustering of thrombotic events in only a small subset of families with thrombophilia. As to the identification of new genetic risk factors for thrombosis, we seem to have arrived at the end of a practicable road with the classical approach of thrombophilia, which usually starts with the study of the association of hemostatic phenotypes and thrombotic risk. At the same time we have undertaken various genetic approaches aiming at identifying polymorphisms/ mutations causing thrombotic risk. This review summarizes what we have learnt so far, what to do and what not to do. The odds for finding remaining common genetic risk factors for venous thrombosis during the next ten years may be predicted to be fairly high.

## Introduction

Thrombophilia is defined as a tendency to develop clots in veins or arteries. Both venous and arterial thrombosis are examples of a complex disease, in which multiple biological pathways contribute to the risk of developing the disease (e.g. blood pressure, blood flow, coagulation, inflammation, atherogenesis). The formation of an occlusive thrombus is the critical event in the acute phase of both diseases; however, the pathogenesis of venous and arterial thrombosis is sufficiently different to consider them as separate diseases. In Western countries thrombophilia is used most frequently in the context of venous thrombosis, to describe a subset of patients with early age of onset, recurrent events, a strong family history of thrombosis, an unusual clinical presentation or the absence of a recognized stimulus (1).

Venous thrombosis is an episodic disease. Present models of venous thrombotic risk hypothesize that a clinical event will occur only when the "thrombosis potential" – which is a function of age, genetic and environmental factors and their interactions (additive or synergistic) – has passed a certain threshold (2). Acquired or environmental risk factors for venous thrombosis include immobilisation, surgery, trauma, use of oral contraceptives and hormone replacement therapy, pregnancy, puerperium and malignancies. One or more of these risk factors are present in 33% of consecutive patients with a first deep-vein thrombosis (Leiden Thrombophilia Study, unpublished observations). The impact of genetic factors on thrombotic risk is also substantial as illustrated by the many reports of familial clustering of thrombophilia (1, 3) and the finding that 23% of consecutive patients with a first venous thrombotic event report at least one first-degree relative with venous thrombosis (4).

During the past 35 years several genetic risk factors for venous thrombosis have been identified by studying families of thrombophilia patients (antithrombin deficiency, protein C deficiency, protein S deficiency, dysfibrinogenemia, APC-resistance associated with factor V Leiden) (5-10). On the other hand, non-O bloodgroup and the prothrombin 20210A mutation were found to be associated with increased risk of venous thrombosis in population based case-control studies (11, 12). Fig. 1 illustrates the progress in our search for genetic risk factors for venous thrombosis. At the end of the year 2000, we can find at least two genetic risk factors in 13% of the thrombophilia families, one genetic risk factor in 60% of the families and no genetic risk factor at all in 27% of the families. Considering the strong support for familial thrombophilia being an oligogenetic disease (13-19), we must conclude that we still lack information on several genetic factors contributing to the risk of venous thrombosis. Many of us expect that a genetic approach to thrombophilia will help us to identify these gene defects.

## The Classical Approach

During the past decades thrombophilia research has focused on the study of the association of thrombosis with functional phenotypes (isolated, persistent and inherited deficiencies or defects) (3, 20). These phenotypes were selected on the basis of their hypothetical effect on fibrin formation and/or degradation in the context of the thrombo-haemorrhagic balance theory. Most of these studies used families recruited through symptomatic probands. Once an association was established between thrombosis and phenotype, the latter served as starting point for the search of the genes involved in its expression. This was a relatively easy task for simple phenotypes such as inherited reduced amounts and/or activity of antithrombin (21, 22), protein C (23-25) and protein S (26, 27). It was more difficult to find the gene responsible for the phenotype of APC-resistance (reduced sensitivity to APC). Careful selection of the candidate gene followed by linkage analysis in a large family with APC-resistance, demonstrated that APC-resistance was completely linked to a marker in the gene coding for factor V and was caused by a mutation in exon 10 of the factor V gene which predicts the replacement of Arg506 by Gln (Factor V Leiden) (10). However, later studies indicated that the sensitivity of a plasma to APC is also possibly influenced by other factor V alleles (R2-allele, R485K) or by non-O blood group alleles (via their effect on plasma factor VIII levels) (28-30). Unfortunately the degree to which these alleles will influence the APC sensitivity ratio is largely dependent on the precise formula of the laboratory test used to measure this phenotype.

More recently, other complex phenotypes have been reported which in population-based case-control studies were found to be associated with increased risk of venous thrombosis: elevated plasma levels of

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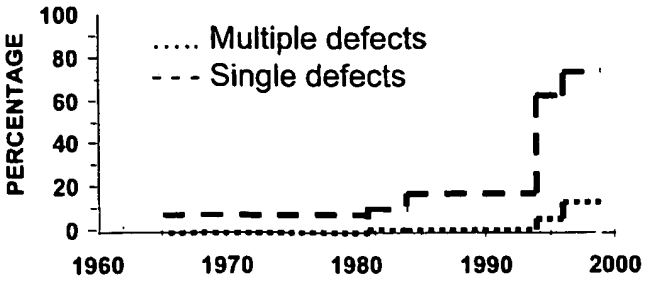


*Fig. 1* Genetic defects in familial thrombophilia. The percentage of thrombophilic families known to have single and multiple genetic defects plotted as function of time. Hallmarks are the years that a new genetic defect was first reported: 1965 (antithrombin deficiency), 1981 (protein C deficiency), 1984 (protein S deficiency), 1994 (factor V G1691A), 1996 (prothrombin 20210A)

homocysteine (31), factor VIII (32), factor XI (33), factor IX (34) and possibly TAFI (35). Finding the genes that contribute to these functional phenotypes will be a major effort and is hardly feasible without a genetic approach. Retrospectively the classical approach for finding genetic risk factors for venous thrombosis has been relatively successful by limiting genetic analysis to only those functional phenotypes that were associated with increased thrombotic risk. As mentioned before, candidate risk-phenotypes have been and still are selected on the basis of our understanding of the regulation of coagulation and fibrinolytic processes. However, the number of candidate phenotypes left is very limited and we have to accept that pathways and proteins outside the coagulation system may be involved in the pathogenesis of venous thrombosis. For the identification of these proteins a genetic approach seems to be more promising.

## The Genetic Approach

Although we seem to have arrived at the end of a practicable road with the classical approach of thrombophilia, we hope that the genetic approach will help us to identify the remaining mutations and polymorphisms contributing to the interindividual variation in thrombotic risk (both risk factors and protecting factors). The general perspective is to generate a list of all the genetic factors that contribute to the development of thrombotic events. Ideally, such a list will help to improve our understanding of the mechanism of thrombus formation in a variety of different environments and to design strategies for treatment and prevention tailored to the genetic profile of the individual (36). Whether the latter objective is meaningful in the context of venous thrombosis is still a matter of debate (37, 38). More discussion and especially more data are needed before we can define the role of genetic testing in the management of thrombophilia (39). Hopefully this debate will end before genetic screening of thrombophilia has become an established routine in our laboratories.

Interestingly, there is an increasing interest in finding genetic factors which influence the response of a patient to oral anticoagulant therapy (40-44). In this context, genetic testing might contribute to a reduction in bleeding risk, and indirectly to the possibility of developing more personalized treatment protocols (duration, intensity and type of treatment).

During the past decade thrombophilia researchers have explored the prospects and feasibilities of a genetic approach and I will try to summarize their achievements below. In the genetic approach, the study of the relationship between particular genotypes and the clinical phenotype precedes the study of the relationships between genotype and functional phenotype and between functional phenotype and clinical phenotype. Of course there are often hybrid approaches in which functional polymorphisms are selected as candidate risk markers.

From the very beginning it was realized that venous thrombosis is a multifactorial disease and that this would add many complications to the genetic analysis of the disease, especially for the identification of thrombosis susceptibility genes in affected families with linkage or allele sharing methods. Fortunately we were well prepared by previous findings. We already knew that mutations in different genes (often coding for proteins in the anticoagulant pathways) can contribute to the same clinical phenotype and that carriers of the same gene mutation can have different clinical phenotypes (deep vein thrombosis, pulmonary embolism, superficial thrombophlebitis, cerebral vein thrombosis, or thrombosis in mesentery or retina) (21, 23, 26, 45). From the study of large collections of families with protein C -, protein S- or antithrombin deficiency, it was also learnt that the penetrance of the disease in mutation carriers is incomplete and largely dependent on the presence of other factors like age, environment or other mutations (21, 25, 26, 45). In general, thrombosis pedigrees will contain individuals who carry a disease allele but are still asymptomatic. On the other hand, we know

*Table 1* Genetic risk factors for venous thrombosis

| risk factor | prevalence (%) | | |
|---|---|---|---|
| | healthy* controls | consecutive* patients | selected** patients |
| factor V 1691A | 3 | 20 | 45 |
| prothrombin 20210A | 2.3 | 6.2 | 18 |
| protein C deficiency | 0.8 | 3.1 | 5.7 |
| protein S deficiency | 1.3 | 1.1 | 5.7 |
| antithrombin deficiency | 0.2 | 1.1 | 4.3 |
| non-O blood group | 57 | 73 | - |

*Data are derived from the Leiden Thrombophilia Study on 474 consecutive patients with a first deep vein thrombosis and 474 healthy controls (12, 32, 95, 128). In this study, protein S deficiency was defined by a protein S level below the 5th percentile of the distribution in the controls.

** Probands of families with thrombophilia (129).

that in such families there will also be individuals who have thrombosis but are not carriers of the private mutation(s) of that family (phenocopies). In these individuals, the thrombotic event might be triggered by an accumulation of (strong) environmental risk factors.

Reviewing the known genetic risk factors for thrombosis and their frequency in the general population, we recognize the existence of "loss of function" mutations and "gain of function" mutations (Table 1). "Loss of function" mutations result in deficiencies of protein C, protein S and antithrombin; many different mutations have been identified in these genes (46-48), all resulting in reduced activity or mass of the encoded protein in plasma. The frequency of these disease alleles in the general population is low (≤0.3% for each of the three forementioned defects). On the other hand, "gain of function" mutations associated with thrombosis risk concern single point mutations which are quite common in the general population. Risk alleles which are common may confound the inheritance pattern in families studied by linkage analysis by introducing independent copies of the disease allele. An example of this comes from the study of Zöller and Dahlbäck in which the authors tried to link the phenotype of APC resistance to the factor V gene (49). Subsequent genotyping of all family members for the factor V Leiden mutation revealed that three independent copies of this allele were segregating in this family, probably as a result of the high frequency of this allele in the Swedish population.

As to the still unknown genetic risk factors for thrombosis we must be prepared to expect both (extremely) rare and rather common risk alleles. Recognition of this heterogeneity should guide us in the selection of suitable experimental approaches; family studies are useful for the identification of rare but relatively strong risk alleles, while case-control studies can be used to identify common (and generally also weaker) risk factors.

## Oligogenic Inheritance of Venous Thrombosis

As to the inheritance of thrombophilia, it has been recognised from the beginning that the disease is found in both men and women, and in successive generations (including male to male transitions), suggesting autosomal dominant inheritance. This was later confirmed by the identification of families with antithrombin-, protein C-, or protein S deficiency. It appeared that familial thrombophilia was an autosomal dominant trait with incomplete penetrance and that it concerned a collection of monogenetic diseases. Only recently has this view changed and now we accept the view that familial thrombophilia is an oligogenetic disease.

There is strong support for the statement that, in thrombophilia families, individuals with two genetic defects will have thrombotic events more frequently and earlier in life than their relatives with a single defect (14-17, 50-52). In addition, Koeleman et al. reported that two locus linkage analysis supported the assumption that the factor V gene and the protein C gene were the two trait loci responsible for the thrombophilia in six families in which both the factor V Leiden allele and a defective protein C allele were segregating (14). This finding explained previous observations that the frequency of the factor V Leiden allele among symptomatic probands of protein C deficient families is much higher than expected on the basis of its population frequency. It also explained why the penetrance of thrombosis is low in protein C deficient family members of an asymptomatic protein C deficient healthy individual (absence of other genetic defects) (53, 54), but will increase after introduction of the factor V Leiden allele in the pedigree (55).

There is also strong support for epistatic interactions between the factor V Leiden allele and protein S gene defects (15, 17). In these studies, almost 40% of the symptomatic probands of protein S deficient thrombophilia families also carry the factor V Leiden mutation. However, Castaman et al. reported the complete absence of factor V Leiden in 16 Italian protein S deficient families (126). In 30% of these families the prothrombin 20210A allele was also segregating (and contributing to the thrombotic risk). Interaction between factor V Leiden and the prothrombin 20210A mutation is highly likely given the 10% prevalence of the latter mutation among symptomatic probands of thrombophilic factor V Leiden families (58, 127). However a formal study of thrombosis families in which both gene defects segregate is still lacking. Interaction between factor V Leiden and antithrombin gene defects has also been reported (16). Notable is the special situation where both gene defects are located on the same chromosome, and cosegregate in the family (16). Interestingly, not all combinations of genetic risk factors show interaction. Especially, there seems to be no interaction between the prothrombin mutation and protein C gene defects (56-58). There are also unexpected interactions such as those between the factor V Leiden allele and the factor V R2 allele (59) or a factor V null-allele (pseudohomozygous APC-resistance) (60-63). Together these recent studies have provided a genetic model for familial thrombophilia that can be used in the design of future genetic studies.

## Candidate Genes

Usually, several biological cascades can be identified that may contribute to the pathogenesis of a disease. This is also true for venous thrombosis where we can recognize the pro- and anticoagulant cascades and the pro- and antfibrinolytic cascades. Of course, each of these cascades has multiple interactions with other cascades as to the regulation of the concentration and activities of its individual components. Genes coding for proteins in these cascades form a first target (candidate genes) in the search for mutations/polymorphisms that may cause a functional phenotype associated with venous thrombosis. This explains the interest of researchers in finding single nucleotide polymorphisms (SNPs) in candidate genes for cardiovascular disease that subsequently can be tested in association or family studies (64, 65).

The main focus of these efforts is directed on the coding regions and promoters of these genes, because these regions are expected to contain most of the SNPs/polymorphisms that contribute to interindividual variation in (plasma) concentration and/or activity of these proteins. Because genotyping by use of the polymerase chain reaction has become a standard technique in most of the clinical laboratories, testing of the effect of SNPs on thrombotic risk in case-control studies has become very popular. As a result, the literature is full of conflicting and/or inconclusive results that subsequently can be used in meta-analyses (for discussion see ref. 66). It is clear that much more attention should be given to the design and interpretation of these association studies (67-69). Finding a higher frequency of an SNP in the patient group than in the control group can be a falsely positive finding, but there are also falsely negative findings. In the case of a true positive finding, it can be that the tested SNP itself causes the increased risk or that it is in linkage disequilibrium (LD) with another SNP which causes the risk. On the other hand, a negative result for an SNP in a particular gene does not mean that this gene is not involved in the pathogenesis of the disease. It may be that the SNP is not sufficiently linked to the disease-causing SNP or that the selected phenotype is too broad (relative risk too low).

Many of the SNPs that have been tested for their effect on venous thrombosis are common in the population (>10%). For these SNPs, it is possible to obtain reliable results from relatively small but properly designed case-control studies. However, to test the effect of less common SNPs (<5%), very large case-control studies may be needed to obtain a significant result (69). To study the effect of SNPs that are rare in the population but good candidate mutations, family studies might be more appropriate.

## Identification of Prothrombotic Mutations by Sequencing of Candidate Genes

At the end of the 1980s, researchers realised that the classical approach of finding genetic risk factors for thrombosis would not work for some candidate genes because it was not possible to measure their expression levels in the blood compartment (e. g. tissue factor, thrombomodulin, TFPI). Therefore, the genes coding for these proteins were sequenced in panels of selected thrombosis patients. We have used this approach to study the presence of prothrombotic mutations in the genes coding for TFPI, tissue factor, thrombomodulin and prothrombin. No candidate mutations were found in the first three genes in a panel of 28 symptomatic probands from families with unexplained thrombophilia. In 1985, Ohlin and Marlar reported the results of their analysis of a similar panel of 28 patients for mutations in the thrombomodulin gene (70). They found one patient heterozygous for a candidate mutation (Asp468Tyr). Since then, these authors have extended their analysis of the thrombomodulin gene to more patients and controls. Four additional candidate mutations have been observed, only one of which (Glu163Glu!) seems to segregate with thrombosis in the family (71). The previously reported Asp468Tyr polymorphism was also observed in one American control (71), but was not found in an Italian population (72).

Unfortunately, there is no information on the effects of these mutations on the biosynthesis, bioavailability or structure/function of the protein. Overall, we may conclude that defects in the thrombomodulin gene are rare and possibly as heterogeneous as in other deficiency states. Whether they are related to venous thrombosis is still an unanswered question. Interestingly, a common SNP in the thrombomodulin locus was found to increase the risk of myocardial infarction (especially when combined with classical risk factors as smoking) (73, 74). Whether this points to a causative role of this thrombomodulin allele in arterial thrombosis is not clear. Because of the small size of the TM gene, the SNP could easily be linked to a functional SNP in another gene.

More successful was the candidate gene approach in the case of the prothrombin gene. Sequencing of the coding and flanking regions of the prothrombin genes of 28 unrelated probands of thrombophilic families revealed a new polymorphism in the 3'UT region of the gene (20210 G/A) (12). Heterozygosity for the 20210A allele was found in 18% of the selected thrombosis patients and only in 1% of healthy subjects. Subsequent genotyping of a population-based case-control study on venous thrombosis (the Leiden Thrombophilia Study) revealed that the 20210A allele was present in 6.2% of the patients and 2.3% of the controls (12). This study nicely demonstrates that narrowing the clinical phenotype from consecutive first deep-vein thrombosis to familial thrombophilia results in an increased frequency of the risk allele (and relative risk). Variations in patient selection may therefore explain the range of Odds ratios for venous thrombosis (OR 2.0-6.6) reported from other centers for the 20210A allele (reviews [56, 75, 76]). So far, there is only one report which could not confirm that the 20210A allele is associated with venous thrombotic risk (77). The initial observation by Poort et al. that the 20210A allele is associated with a 30% increase in plasma prothombin levels has also been confirmed in other centers. The observation made in the Leiden Thrombophilia Study that elevated plasma prothrombin levels (>115 U/dl) were associated with a 2.1 fold increased risk of thrombosis demonstrated that the 20210A allele acts on thrombotic risk via increased plasma prothrombin levels (gene → risk, gene → functional phenotype, functional phenotype → risk). Whether the 20210 G→A mutation causes the elevation in plasma prothrombin is not yet clear. In vitro studies need to answer the question of whether the G→A transition in nt 20210 causes increased transcription, polyadenylation or translation efficiency. Haplotype analysis of 20210 A carriers indicates strong linkage disequilibrium and suggests a founder haplotype (78). In our laboratory, we have used six prothrombin gene polymorphisms for haplotyping 22 homozygous 20210A carriers from different parts of the world. All 20210A alleles were found to have the same haplotype (3728T-4125C-8845G-9832T-19911A-20210A) (79). Like the Factor V Leiden allele, the prothrombin 20210A allele is extremely rare in non-Caucasians (80).

A more recent example of the candidate gene approach, is the work of Merati et al. (81) on the gene coding for the endothelial protein C receptor (EPCR). This is a transmembrane protein expressed in endothelial cells of large vessels that together with thrombomodulin is involved in the activation of protein C (for a review see ref 82). Systematic analysis of exonic regions in the EPCR genes of thrombosis patients revealed a rare sequence variation in exon 3. It concerns an insertion of 23 nucleotides preceding the insertion point (nt4031), which introduces a frameshift and premature stop (81). Because it is expected that the truncated protein encoded by this allele will lack the cytoplasmatic tail, transmembrane domain and part of the extracellular domain, this mutation is probably a good model for an EPCR null-allele. Unfortunately, its allele frequency is low both in patients and contols (≤1%), so that it will be difficult to investigate its association with thrombotic risk in case-control studies. In our laboratory we have identified one heterozygote for this insertion among 50 patients with thrombophilia. Family studies might help to reveal the clinical phenotype of this mutation.

## Polymorphisms and Thrombotic Risk

As mentioned above, testing SNPs for their effects on risk for a particular disease phenotype is also very popular in thrombophilia research. Most of these SNPs seem to be functional in the sense that at least an association has been found with levels or activity of the affected protein. However it is often difficult to assess whether the SNP itself is the cause of the functional phenotype or whether it is in linkage disequilibrium with the functional mutation (64, 66, 83). Also, there are examples where several polymorphisms in the same gene contribute to the functional phenotype. For instance, polymorphisms in the promoter (84-86), coding region (87, 88) and intronic regions (89) of the factor VII gene all independently seem to influence the plasma factor VII levels (90). In such a situation it seems better to use dense haplotypes in the association studies. This is also the best approach when there is no functional SNP known in the gene under investigation. Using a single or even a few SNPs to study the effect of a gene on the risk of disease is not recommended, given that in the general population useful linkage disequilibrium might not extend beyond an average distance of 3 kb (83). However, the actual distance at which disequilibrium can be observed is largely dependent on the age of the SNP and may be as large as 100 kb (91).

*Table 2* Polymorphisms in candidate genes; effects on levels, function and thrombotic risk (I)

| gene | polymorphism[1] | association with | | effect on thrombotic risk | | comments |
|---|---|---|---|---|---|---|
| | | levels | function | yes | no | |
| tissue factor | -1208 D/I[130] | yes (↓)[130] | - | reduction[130] | - | I: insertion 18nt |
| factor VII | Arg 353 Gln[87] | yes (↓)[87,85,90,131] | - | - | no[132] | - |
| factor X | -343 D/I<br>-222 C/T<br>-220 C/A | no[133] | - | - | no[133] | I: insertion TTGTGA |
| factor X | -40 C/T[134] | no[133] | - | - | no[133] | - |
| factor XII | 46 C/T[135] | yes (↓)[135] | - | - | - | - |
| factor II | 19911 G/A[79] | yes (↑)[79] | - | - | no[79] | intron 13 |
| factor V | Arg 485 Lys[136] | - | yes?[29] | ?[137] | - | APC-resistant?[29] |
| factor V | His1299 Arg[28] | yes (↓)[28,138,140] | yes?[141] | increase[59,139] | no[138,138] | marker for the R2-FV allele |
| VWF | -1234 C/T[142] | yes (↑)[142], no[143] | - | - | no[143] | marker for VWF promoter allele |
| fibrinogen Aα | Thr 312 Ala[145] | - | ?[144] | ?[144] | - | only for pulmonary embolism? |
| fibrinogen Bβ | -455 G/A | yes (↑)[148-148] | - | - | no[132] | Hae III restriction site |
| factor XIII | Val34Leu[152] | no[156,160] | yes[156-159] | reduction[150,151] | no[149,153-156,160] | - |
| TAFI | -438 G/A[161] | yes (↓)[161] | - | reduction?[161] | no[161] | marker for TAFI promoter allele |
| TAFI | -152 A/G | no[161] | - | - | no[161] | - |

[1] The allele in bold was tested for its effect on level, function or thrombotic risk. References are given as indices in each column

Tables 2 and 3 provide a summary of the SNPs/polymorphisms which have recently been used in the study of thrombophilia. Not included in these Tables are factor V G1691A (factor V Leiden), prothrombin 20210A and MTHFR C677T. The association or lack of association of these genotypes with venous thrombotic risk has been amply documented in the literature (for recent reviews see refs 92, 56, 93 and 197).

Presently, there is no firm support for an effect of the MTHFR 677A allele on the risk of venous thrombosis, either in the absence or presence of factor V Leiden (93, 94); it is surprising therefore that this polymorphism is still frequently included in genetic studies of thrombophilia. Furthermore, it is interesting to see that SNPs in the genes coding for factor VII, fibrinogen, PAI-I, and tPA receive much less attention in the study of thrombophilia than they do in the study of arterial thrombosis (review [92]).

All studies have investigated the effect of a particular SNP on the risk of venous thrombosis. There are, however, considerable differences in the selection of patients; first events/recurrent events, consecu-

*Table 3* Polymorphisms in candidate genes; effects on levels, function and thrombotic risk (II)

| gene | polymorphism[1] | association with | | effect on thrombotic risk | | comments |
|---|---|---|---|---|---|---|
| | | levels | function | yes | no | |
| protein C | -1641G/A,-1654C/T[162] | yes (↓)[162,163,164] | - | increased?[162,163] | - | GC homozygotes versus AT homozygotes |
| protein S | A 2148 G[165] | yes (↓)[166,167] | - | - | - | Pro 626 dimorphism |
| protein S | C 2698 A[168] | yes (↑)[167] | - | - | - | in 3'UT region |
| thrombomodulin | Ala 455 Val[169] | - | - | - | no[169] | - |
| EPCR | 4031 I/D[82] | - | - | increased[82] | - | insertion 23 bp in exon 3 |
| TFPI | -287 T/C[174] | yes (↓)[174] | - | - | - | - |
| TFPI | -399 C/T[171] | no[171,174] | - | - | no[171] | - |
| TFPI | Val264Met[175] | yes (↓)[175] | - | - | no[172] | - |
| TFPI | T 384 C[175] | no[175] | - | - | - | Tyr 57 dimorphism |
| TFPI | Pro151Leu[170] | - | - | increased[170] | no[173,176,177] | - |
| tissue plasminogen activator | I/D intron h[178] | yes[179,180,181] | - | - | - | - |
| plasminogen activator inhibitor | -675 4G/5G[183] | yes[182,183,186-189] | - | *increased[185,194] | no[184,185,190,192,193] | *in carriers FV Leiden or protein S gene defect |
| | -844 A/G[184,191] | no[191] | - | *increased[184] | - | *in carriers FV Leiden |
| protease activated receptor -1 | -506 I/D[195] | - | - | decreased[195] | - | only in men |
| | -1426 C/T[195] | - | - | - | no[185] | - |
| | IVS -14 A/T[195] | - | - | - | no[195] | |
| Angiotensin I converting enzyme | I/D(intron16)[196] | yes (↑)[104,196] | - | increased[104] | - | insertion 287 bp |

[1] The allele in bold was tested for its effect on level, function or thrombotic risk. References are given as indices in each column

tive patients/selected patients, deep-vein thrombosis/all thrombotic events. Of course this may influence the outcome of the studies as well as the extent of agreement that can be obtained between studies of the same genotype in different centers. Also, a polymorphism might differ in its effect on different phenotypic expressions of venous thrombosis. For example, factor V Leiden is a risk factor for deep-vein thrombosis (95), cerebral vein thrombosis (96), superficial vein thrombosis (97) and portal vein thrombosis (98), but not for primary pulmonary embolism (99) and retinal vein thrombosis (100). Finally, differences in exposure to environmental factors may influence the results. The use of oral contraceptive enhances the risk of factor V Leiden and prothrombin 20210 A alleles (101, 102), while there is no interaction between these alleles and surgery (103). Thus, Factor V Leiden is not an important risk factor for postoperative thrombosis in patients undergoing hip arthroplasty, while the D allele of the polymorphism in intron 16 of the gene coding for the angiotensin-I converting enzyme increases the risk almost tenfold (104). The latter polymorphism had only a weak effect on thrombotic risk in a small case control study in African-Americans, but only in men (105). This result can possibly be explained by the observation that surgery is more frequently found in men than in women to be a precipitating factor for a thrombotic event.

A final observation that can be made from Table 2 is that there is a growing interest in polymorphisms that protect against venous thrombosis (e. g. factor XIII Val 34Leu, and TAFI -4386 G/A). This mirrors the growing interest in the effect of prothrombotic mutations on the clinical phenotype of congenital and acquired bleeding disorders (106, 107).

## Family Studies

To find susceptibility loci for complex diseases, investigators have studied twins, sib pairs, small nuclear families and large extended pedigrees. In the field of thrombophilia, there is still limited experience with these types of studies, but this situation will probably change rapidly now that the first genome-wide scan in thrombosis families has been recently completed (XVIII ISTH Congress, Paris, 2001).

In the context of this review, I will not discuss the various statistical genetic approaches. During the past decade there has been enormous progress in what these programs can do. Using more of the available information (e. g. multipoint analysis) in both affected and non-affected individuals leads to more statistical power and thus enhances the probability of finding significant effects. Genetic markers can be either chosen in specific candidate genes or be a selection that covers the whole genome at the required density (e. g. with an intermarker distance of 1 cM). Markers should have a high polymorphic information content (e. g. CA repeats) because this will prevent loss of information. Polymorphic markers in or very close to candidate susceptibility genes for thrombosis, have been identified by determining the location of the genes on the linkage map (108, 109).

With regard to thrombophilia, linkage studies might be used to answer two types of questions. First, what are the genetic determinants of a functional phenotype, e. g. a phenotype that has previously been shown to be associated with venous thrombotic risk. It is also possible of course to select a phenotype for which it is unknown whether there is an associated risk. Finding the genotypes that influence such a phenotype may reveal new candidate genes, after which the effect of these genes on thrombotic risk can be explored in the usual association studies. Before actually performing the linkage analysis, it is important to have an estimate of the heritability of the phenotype. For many of the hemostasis and fibrinolysis parameters such heritabilities have been published (110-114). It should however be recognized that observed heritability may be different in different environments or geographical areas.

The second obvious application is to use linkage in thrombosis families to identify thrombosis susceptibility loci. For such studies it is generally important to have an adequate genetic model for the disease. At the present time, we may have such a model for familial thrombophilia (see above). Of course it is also possible to combine both types of studies in the same (thrombosis ) families, in which case it is possible to test for pleiotropic effects of loci on (a) quantitative trait(s) and susceptibility to thrombosis. An example of such a study was recently published by Soria et al. (115). In this study, the authors demonstrated that the prothrombin 20210A mutation jointly influences thrombosis and plasma prothrombin activity. This study was performed within the framework of the GAIT project, a project that focuses on the identification of susceptibility loci for hemostatic phenotypes and thrombosis using multipoint linkage analysis by variance components techniques (116, 117). The published results of this project are very promising and partly agree with views based on the interpretation of findings in association studies; such as the genetic linkage of the ABO locus to plasma VWF, factor VIII, and APTT levels (118). They also agree with the reported significant genetic correlations between thrombosis and VWF/factor VIII, Factor IX, factor XI, the APC sensitivity ratio and homocysteine levels (119). Two comments may be made. First, the authors used a rather broad definition of the clinical phenotype which includes arterial thrombosis. And secondly, many of the hemostatic phenotypes used in this study concern functional phenotypes. Although in general these phenotypes will give more information, results might be reagent specific (e. g. one stage clotting assays, APC sensitivity ratios measured with different methods). For instance high factor VIII levels might cause falsely high readings of other intrinsic factors in one stage assays. Confirmation of the results in different sets of families will therefore be important.

As mentioned above, there is strong support for the hypothesis that familial thrombophilia is an oligogenetic disease. This would mean that in general at least two different genetic defects will segregate in a thrombophilic family. Knowing one gene defect in such a family might offer a good opportunity for finding the second by linkage analysis. This is the approach followed by Bovill and coworkers in their study of the genetic causes of thrombosis in a very large pedigree with protein C deficiency and thrombosis. This family was first reported in 1989 (120), later the authors reported the presence of the private protein C gene mutation (His107 Pro) (121) and concluded that a still unknown genetic defect interacted in this family with the protein C gene defect to increase thrombotic risk (122). Subsequently, they found that the prothrombin 20210A allele was fairly frequent in this family but did not contribute significantly to the thrombotic risk (57). For this analysis they used a transmission/disequilibrium test (123) modified for calculating likelyhoods in pedigrees. In their most recent paper, the authors reported the results of genetic screening of 34 candidate genes for a prothrombotic interaction with the protein C gene defect in an informative subset of the family (109). However, although almost all known hemostatic genes were included (blood group and factor VIII were not included) none of them was implicated as the second prothrombotic gene in this family.

A few years ago we obtained very similar results when we tried to find (a) second gene defect(s) in four protein C deficient families using markers for 24 thrombosis candidate genes (124). A total of 66 individuals were genotyped, 22 of which were affected by thrombosis. We used the TLINK program for the analysis, and assumed that the

protein C gene was one of the two disease genes. No significant lod scores were obtained for any of the tested markers. A marker for the protein C gene itself gave in single locus analysis a lodscore of 1.8 at theta = 0.05!

We appeared to have more success with a very similar approach in a set of thrombophilia families with one known genetic defect (either Factor V Leiden, antithrombin deficiency, or protein S deficiency) (125). In these families, we specifically investigated whether there was support for a second thrombosis susceptibility locus in the neighbourhood of the protein C locus (2q13-14), using nine highly polymorphic markers. This region was difficult to study in the protein C deficient families, while it could not be excluded that in some of the symptomatic protein C deficient families the apparent dominant inheritance of the disease was caused by two different but closely linked defects on chromosome 2. Results of parametric (TLINK) and non-parametric linkage analysis (SIMIBD, GENEHUNTER) provided weak evidence (lodscores 1.7-1.9) for a second thrombosis susceptibility locus in these families (apart from the FV gene, the protein S gene or the antithrombin gene). This locus is located close to the gene coding for interleukin 1. However, sequencing of a number of candidate genes in this region, did not reveal any sequence variation that could constitute the disease determinant. Confirmation of this result in different sets of families is needed before a more systematic analysis of this region is considered.

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Evidence Appendix D

Please Mail Mailing Label



SHORT REPORTS

# Association of idiopathic venous thromboembolism with single point-mutation at Arg$^{506}$ of factor V

*Jan Voorberg, Jeanet Roelse, Rianne Koopman, Harry Büller, Fenny Berends, Jan W ten Cate, Koen Mertens, Jan A van Mourik*

Abnormal coagulation factor V may underlie the thrombotic events associated with resistance to activated protein C (APC). We analysed 27 consecutive patients with documented idiopathic (recurrent) thromboembolism for the occurrence of point mutations within the APC sensitive regions of blood coagulation factor V. In 10 patients we observed a single basepair mutation resulting in a substitution of Arg$^{506}$ to Gln. This mutation was significantly linked to in-vitro resistance to APC in these subjects. This mutation at Arg$^{506}$ of factor V may form the molecular basis for the thrombotic events associated with APC resistance.

*Lancet* 1994; **343**: 1535–36

See *Commentary page 1515*

Venous thromboembolism has been associated with molecular defects in several haemostatic components: antithrombin III, protein C, protein S, plasminogen, and fibrinogen.[1] However, in over 90% of patients the cause remains obscure.[2] A poor anticoagulant response to activated protein C (APC) has been observed in about 20–30% of patients with an idiopathic predisposition to thromboembolic disease.[3,6] This abnormal response has been linked to a plasma factor which appeared to be identical to coagulation factor V.[7] These observations suggest that a molecular abnormality in factor V underlies the thrombotic events that are associated with a defective anticoagulant response to APC in vitro. We report linkage between resistance to APC and a single point-mutation at a putative APC cleavage site at Arg$^{506}$ of factor V.

We investigated 27 consecutive patients (13 men; mean age 53, range 23–79) with (recurrent) idiopathic episodes of thromboembolism confirmed by contrast venography, pulmonary angiography, or both. None of the patients received oral anticoagulants at the time of the study. Patients with cancer or lupus anticoagulants were excluded. No patient had an acquired or inherited deficiency of antithrombin III, protein C, protein S, or plasminogen. Routine screening of blood coagulation and fibrinolysis revealed no abnormality. Resistance to APC was assessed by the APC-dependent prolongation of the activated partial thromboplastin time (Coatest APC Resistance, Chromogenix, Sweden).[3,5] An APC sensitivity ratio $\leq 2{\cdot}0$ was considered to represent a defect in the anticoagulant response to APC.

Patients were analysed for the presence of mutations at Arg$^{506}$ in factor V with the following oligonucleotide primers: 5'CATCACGTTTTCACCTCATCAGG3' (primer 506–2, nucleotides 1708–1730 of human factor V) and 5'ATCAGAGCAGTTCAACCAGGG3' (506–5, nucleotides 1414–1435). RNA was isolated from peripheral blood lymphocytes by the RNAzol B method (WAK Chemie, Bad Homburg, Germany) and cDNA was prepared.[4] Amplification by polymerase chain reaction with primers 506–2 and 506–5 yields a fragment of 316 basepairs, which encodes the part of factor V that contains the APC cleavage site at Arg$^{506}$. The occurrence of mutations at Arg$^{506}$ was monitored by direct sequencing of the amplified fragment.

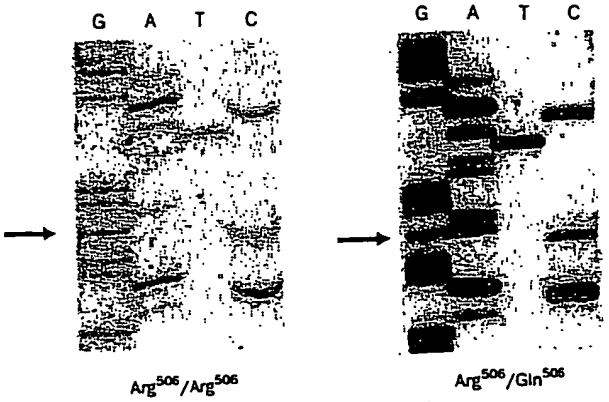


Figure 1: **Sequence analysis of factor V cDNA**

Factor V cDNA derived from patient heterozygous for Arg$^{506}$ to Gln mutation is shown in right panel. Heterozygosity is scored by occurrence of both a "G" and an "A" at second basepair of codon Arg$^{506}$ (CGA/CAA) of factor V (arrow). In left panel, sequence analysis of patient who does not carry the mutation is displayed. Arrow = single "G" observed at second basepair of codon Arg$^{506}$ (CGA/CGA).

Previous studies with bovine factor V have shown that APC partly inactivates factor V by cleavage at the peptide-bond Arg$^{505}$-Gly$^{506}$.[9] Direct sequencing of the corresponding part of factor V cDNA derived from our patients revealed a single G to A transition, which results in substitution of Arg$^{506}$ for Gln (figure 1, right panel). 10 of the 27 patients were heterozygous for the Arg$^{506}$ to Gln$^{506}$ mutation. 8 of the 27 (30%) had an abnormal APC sensitivity ratio ($\leq 2{\cdot}0$), in agreement with the frequency in similar cohorts.[3,5] The abnormal APC sensitivity ratio was significantly linked to the Arg$^{506}$ to Gln mutation (figure 2; *U* test, $p < 0{\cdot}0001$). In 3 patients who were heterozygous for the Arg$^{506}$ Gln mutation, APC ratio was just above 2·0, and in only 1 patient did an abnormal APC sensitivity ratio (1·9) coincide with the normal Arg$^{506}$/Arg$^{506}$ genotype.

Our results indicate that APC resistance in patients with idiopathic thromboembolism was linked to a single mutation at the putative APC cleavage site at Arg$^{506}$ in factor V. The data suggest that APC resistance is not due to a deficiency of a cofactor of APC as has been proposed,[3,6,7,10]

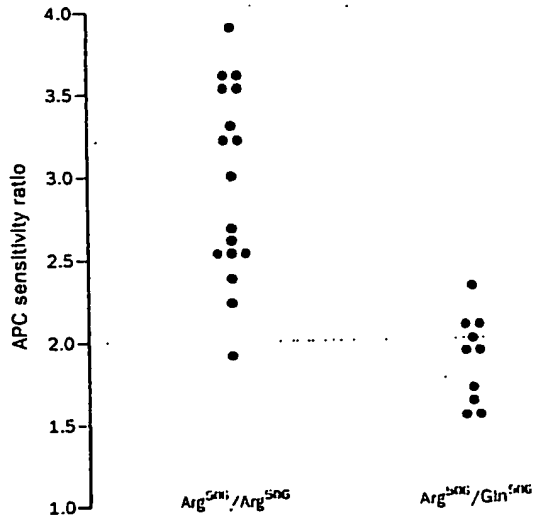


Figure 2: **Anticoagulant response to APC**

Right = 10 patients heterozygous for Arg$^{506}$—Gln mutation, and left = 17 patients who do not carry the mutation.

but merely reflects the inability of APC to inactivate the pro-coagulant factor V. The high frequency of APC resistance in patients with idiopathic venous thromboembolism suggests that the mutation $Arg^{506}$ to Gln may be a major cause of inherited thrombophilia.

We thank Dr W Schaasberg and Prof W G van Aken, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, for doing statistical analysis and for support throughout the study, respectively.

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# Linkage between inherited resistance to activated protein C and factor V gene mutation in venous thrombosis

*Bengt Zöller, Björn Dahlbäck*

Resistance to activated protein C (APC) is a major cause of familial thrombophilia, and can be corrected by an anticoagulant activity expressed by purified factor V. We investigated linkage between APC resistance and the factor V gene in a large kindred with familial thrombophilia. Restriction fragment length polymorphisms in exon 13 of the factor V gene were informative in 14 family members. The 100% linkage between factor V gene polymorphism and APC resistance strongly suggested a factor V gene mutation as a cause of APC resistance. A point mutation changing $Arg^{506}$ in the APC cleavage site to a Gln was found in APC resistant individuals. These results suggest factor V gene mutation to be the most common genetic cause of thrombophilia.

*Lancet* 1994; **343**: 1536–38

*See Commentary page 1515*

Heterozygous protein C or protein S deficiency is associated with familial thrombosis,[1] and inherited resistance to activated protein C (APC) as a possible cause of thrombophilia was discovered in a family with thrombosis.[2] It is now well established that APC resistance is a major cause of venous thrombosis,[3–6] and APC resistance in different families appears to be characterised by a molecular similarity.[3] In a cohort of thrombosis patients, APC resistance was at least ten times more frequent than any of the other anticoagulant protein deficiencies (40% *vs* ≤4%) and in familial thrombophilia, it accounted for more than 50% of cases.[3] APC resistance in the general population is around 5%.[3,5]

We have isolated and characterised the protein that corrects APC resistance, and found it to be identical to factor V.[7] Factor V is pro-coagulant after activation by thrombin, whereas the novel anticoagulant cofactor activity, which we have also found in purified systems (Dr L Shen and BD, Department of Clinical Chemistry, Malmo General Hospital, Sweden), appears to be a property of unactivated factor V. Because APC-resistant plasma contains normal levels of factor V pro-coagulant activity, APC resistance may be caused by mutations in the factor V gene resulting in selective loss of the anticoagulant activity of factor V or in increased resistance to APC of mutant factor Va itself. We have investigated whether APC resistance is due to mutation in the factor V gene in a study of linkage in a large kindred with familial thrombophilia.

The APC resistance test, a modified activated partial thromboplastin time in which the anticoagulant response to standardised addition of APC is measured, was done as described.[2,3] The results were expressed as the APC ratio (clotting time with the APC/$CaCl_2$ solution divided by clotting time with $CaCl_2$). Family members with confirmed APC ratios under 2·0 were considered to be APC resistant.[3] Free and total protein S were measured with a radioimmunoassay.[3] Family members with a concentration of free protein S below the normal limit were considered to be protein S deficient. Their total protein S levels were slightly low or in the low normal range.

Genomic DNA was prepared from EDTA-blood by standard procedures. A sequence of 1188 basepairs (bp) of the factor V gene (nucleotides 2066 to 3254 of the factor V cDNA; sequence from Genebank) was amplified from genomic DNA with two primers 5'GAACTTGGATGTTAACTTCC3' and 5'GGCTTCACT-TCTTAGAGGGTG3' (figure). The conditions for polymerase chain reaction (PCR) for 40 cycles of amplification were: 60 s denaturation at 93°C, 30 s annealing at 61°C, and 180 s extension at 72°C. After amplification, the DNA was cleaved with *Taq*1 and with *Eco*R1, and subjected to agarose-gel electrophoresis. The region in exon 10 that encodes one of the APC cleavage sites in factor V was PCR amplified from genomic DNA with 5'GGG-CTAATAGGACTACTTCTAATC3' (corresponding to $Gly^{490}$-$Ile^{497}$) and 5'TCTCTTGAAGGAAATGCCCCATTA3' (derived from intron sequence provided by Dr W Kane). The PCR conditions were 5 min initial denaturation at 94°C followed by 30 cycles of 60 s denaturation at 93°C, 30 s annealing at 61°C, and 90 s

RELATED PROCEEDINGS APPENDIX

This appendix includes copies of the decisions in the following proceedings related to the present application:

1. Final Judgment for Patent Interference No. 105,235.
2. Final Judgment for Patent Interference No. 105,268.
3. Final Judgment for Patent Interference No. 105,269.

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Paper 69
Filed: June 30, 2006

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Fax: 571-273-0042

UNITED STATES PATENT AND TRADEMARK OFFICE

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BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES

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JOHN H. **GRIFFIN**,
SAMUEL I. RAPAPORT, and DZUNG T. LE,

Junior Party
(Patent 5,834,223),

v.

BJORN **DAHLBACK**

Senior Party
(Application 08/500,917).

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Patent Interference No. 105,268

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Before Lorin, Lane, and Tierney, Administrative Patent Judges.

Lane, Administrative Patent Judge.

**Final Judgment - No interference-in-fact- Bd.R. 127**

Upon consideration of the record of the interference, it is

ORDERED that there is no interference-in-fact between the involved claims of Griffin's 5,834,223 patent and the involved claims of Dahlback's 08/500,917 application;

FURTHER ORDERED that if there is a settlement agreement the parties are directed to 35 USC § 135(c) and Bd.R. 41.205; and

FURTHER ORDERED that this Judgment shall be entered into the administrative record of Griffin's 5,834,223 patent and Dahlback's 08/500,917 application.

/ss/ Hubert C. Lorin
HUBERT C. LORIN
Administrative Patent Judge

/ss/ Sally Gardner Lane
SALLY GARDNER LANE
Administrative Patent Judge

BOARD OF PATENT APPEALS AND INTERFERENCES

TIERNEY, Administrative Patent Judge, dissenting.

For the reasons set forth in my dissent from the Decision on Motions (Paper 68), I likewise dissent from the judgment of no interference-in-fact.

/ss/ Michael P. Tierney
MICHAEL P. TIERNEY
Administrative Patent Judge

) BOARD OF PATENT
) APPEALS AND
) INTERFERENCES

cc (via Overnight Mail):

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Paper 73
Filed: June 30, 2006

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Fax: 571-273-0042

UNITED STATES PATENT AND TRADEMARK OFFICE

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BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES

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JOHN H. **GRIFFIN**,
SAMUEL I. RAPAPORT, and DZUNE T. LE,

Junior Party
(Patent 6,083,757),

v.

BJORN **DAHLBACK**

Senior Party
(Application 08/500,917).

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Patent Interference No. 105,269

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Before Lorin, Lane, and Tierney, Administrative Patent Judges.

Lane, Administrative Patent Judge.

**Final Judgment - No interference-in-fact- Bd.R. 127**

Upon consideration of the record of the interference, it is

ORDERED that there is no interference-in-fact between the involved claims of Griffin's 6,083,757 patent and the involved claims of Dahlback's 08/500,917 application;

FURTHER ORDERED that if there is a settlement agreement the parties are directed to 35 USC § 135(c) and Bd.R. 41.205; and

FURTHER ORDERED that this Judgment shall be entered into the administrative record of Griffin's 6,083,757 patent and Dahlback's 08/500,917 application.

| | | |
|---|---|---|
| /ss/ Hubert C. Lorin | ) | |
| HUBERT C. LORIN | ) | |
| Administrative Patent Judge | ) | |
| | ) | |
| | ) | BOARD OF PATENT |
| /ss/ Sally Gardner Lane | ) | APPEALS AND |
| SALLY GARDNER LANE | ) | INTERFERENCES |
| Administrative Patent Judge | ) | |

TIERNEY, Administrative Patent Judge, dissenting.

For the reasons set forth in my dissent from the Decision on Motions (Paper 72), I likewise dissent from the judgment of no interference-in-fact.

| | |
|---|---|
| /ss/ Michael P. Tierney | ) BOARD OF PATENT |
| MICHAEL P. TIERNEY | ) APPEALS AND |
| Administrative Patent Judge | ) INTERFERENCES |

cc (via Overnight Mail):

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Paper 69

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UNITED STATES PATENT AND TRADEMARK OFFICE

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BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES

---

JOHN H. **GRIFFIN**,
SAMUEL I. RAPAPORT, and DZUNG T. LE,

Junior Party
(Patent 5,705,395),

v.

BJORN **DAHLBACK**

Senior Party
(Application 08/500,917).

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Patent Interference No. 105,235

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Before Lorin, Lane, and Tierney, Administrative Patent Judges.

Lane, Administrative Patent Judge.

**Final Judgment - No interference-in-fact- Bd.R. 127**

Upon consideration of the record of the interference, it is

ORDERED that there is no interference-in-fact between the involved claims of Griffin's 5,705,395 patent and the involved claims of Dahlback's 08/500,917 application;

FURTHER ORDERED that if there is a settlement agreement the parties are directed to 35 USC § 135(c) and Bd.R. 41.205; and

FURTHER ORDERED that this Judgment shall be entered into the administrative record of Griffin's 5,705,395 patent and Dahlback's 08/500,917 application.

/ss/ Hubert C. Lorin
HUBERT C. LORIN
Administrative Patent Judge

/ss/ Sally Gardner Lane
SALLY GARDNER LANE
Administrative Patent Judge

BOARD OF PATENT APPEALS AND INTERFERENCES

TIERNEY, Administrative Patent Judge, dissenting.

For the reasons set forth in my dissent from the Decision on Motions (Paper 68), I likewise dissent from the judgment of no interference-in-fact.

| | |
|---|---|
| /ss/ Michael P. Tierney | ) BOARD OF PATENT |
| MICHAEL P. TIERNEY | ) APPEALS AND |
| Administrative Patent Judge | ) INTERFERENCES |

cc (via Overnight Mail):

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